Protein kinases in myocardial protection

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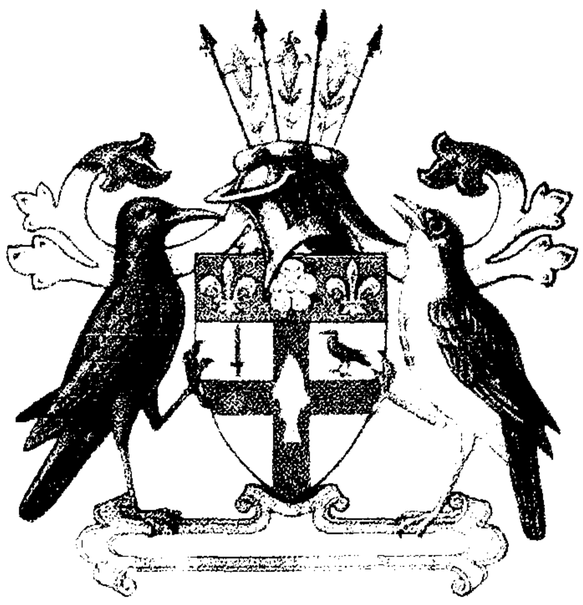

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ABSTRACT

Background

A strategy for protecting the myocardium during ischaemia would reduce the mortality associated with ischaemic heart disease. Ischaemic preconditioning (IP) refers to the resistance to ischaemic injury that follows brief periods of ischaemia and reperfusion. A better understanding of the signalling pathways that underlie preconditioning may enable protection without the risk of an ischaemic trigger.

Aims

To examine the role of protein kinases in the intracellular signalling pathway responsible for protection following ischaemic preconditioning.

Results

Protein kinase C (PKC) activation is critical for protection, although the isoform responsible is unknown. We used PKCε deficient mice to study the role of this PKC isoform in IP. The hearts from mice lacking PKCε (-/-) and matched heterozygous mice (+/-) were preconditioned by 4 × 4 minutes ischaemia/6 minutes reperfusion. In (+/-) hearts, IP significantly reduced infarction and improved contractile recovery. In contrast, there was no difference in infarction between IP and control hearts from (-/-) mice, although IP still enhanced contractile recovery in these hearts. These data suggest that PKCε is essential for the reduction in infarct size seen following IP, but is not associated with the improvement in contractile recovery.

Mitogen-activated protein kinases (MAPKs) have been implicated downstream of PKC. Of all the MAPK isoforms, only p38 was activated during simulated ischaemia in neonatal rat cardiac myocytes. Surprisingly, this activity was diminished in cells protected by preconditioning. Moreover, pharmacological inhibition of p38 protected myocytes during simulated ischaemia. Ectopically expressed p38α was activated during ischaemia and inhibited by preconditioning, whereas p38β was inhibited during ischaemia. Specific inhibition of p38α, using a dominant negative mutant, also protected myocytes against simulated ischaemia.

Conclusions

These data suggest that PKCε is the isoform responsible for preconditioning against infarction in the isolated mouse heart. The enhanced recovery after preconditioning in PKCε deficient mice, suggests that protection against infarction and contractile recovery maybe mutually exclusive. In neonatal rat myocytes, p38α MAPK is activated during simulated ischaemia, which aggravates cell injury. The inhibition of p38α activation that follows ischaemic preconditioning may, at least in part, contribute to the resulting protection.

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ABBREVIATIONS

2×SB	Two-times sample buffer
5-HD	5-hydroxydecanoate
aa	Amino acid
ACE	Angiotensin converting enzyme
ANCOVA	Analysis of co-variance
ANOVA	Analysis of variance
APD	Action potential duration
aPKC	Atypical protein kinase C
APS	Ammonium persulphate
ATP	Adenosine triphosphate
BMK1	Big mitogen-activated protein kinase-1
bp	Base pair
bpm	Beats per minute
CK	Creatine kinase
cPKC	Conventional protein kinase C
DAG	Diacylglycerol
DC-Chol	3β(N- (N', N'-dimethylaminoethane) carbomyl)-cholesterol
DI H_2O	De-ionised water (resistivity >18mΩ/cm^3)
DMEM	Dulbecco's modified Eagle's medium
DN	Dominant negative
DNA	Deoxyribonucleic acid
DOPE	Dioleoyl L-α-phosphatidylethanolamine

EDTA	Ethylenediaminetetra-acetic acid disodium salt
EGF	Epidermal growth factor
ES	Embyonic stem
ERK	Extracellular signal-regulated kinase
FCS	Foetal calf serum
FGM	Full growth media
GDP	Guanosine diphosphate
GFP	Green fluorescent protein
G-protein	Heterotrimeric GTP-binding protein
GPCR	G-protein coupled receptor
GTP	Guanosine triphosphate
HEK 293	Human embryonic kidney, clone 293 (cell line)
HSP	Heat shock protein
IB	Ischaemia buffer
IFN	Interferon
IL	Interleukin
IMS	Inter-membrane space
iNOS	Inducible nitric oxide synthase
IP_3	Inositol 1,4,5-triphosphate
i.p.	Intra-peritoneal
IP	Ischaemic preconditioning
IRES	Independent ribosomal entry site
JNK	C-Jun N-terminal kinase
K_{ATP}	ATP-sensitive potassium channel

Kb	Kilobase
KDa	Kilodalton
K-H	Krebs-Henseleit
Kir	Inward rectifying potassium channel
LB	Luria-Bertani
LDH	Lactate dehydrogenase
LVDP	Left ventricular developed pressure
MAPK	Mitogen-activated protein kinase
MAPKAPK2	Mitogen-activated protein kinase-activated protein kinase 2
MCS	Multiple cloning site
MitoK_{ATP}	Mitochondrial K_{ATP} channel
MKK	Mitogen-activated protein kinase kinase
MKP	Mitogen-activated protein kinase phosphatase
MnSOD	Manganese superoxide dismutase
MOI	Multiplicity of infection
MPG	N-2-mercaptopropionylglycine
mTOR	Mammalian target of rapamycin
MTT	3-(4,5-dimethylthiaziazol-2-yl)2,5-diphenyl tetrazolium bromide
Mw	Molecular weight
NADH	Nicotinamide adenine dinucleotide
NGF	Nerve growth factor
nPKC	Novel protein kinase C
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline

PCR	Polymerase chain reaction
PDK1	Phosphoinositide-dependent kinase-1
pfu	Plaque forming unit
PI3-kinase	Phosphatidylinositol 3-kinase
PIP_2	Phosphatidylinositol 4,5-biphosphate
PKA	Protein kinase A
PKB	Protein kinase B
PKC	Protein kinase C
PLC	Phospholipase C
PLD	Phospholipase D
PMA	Phorbol 12-myristate 13-acetate
PPase	Protein phosphatase
PS	Phosphatidylserine
PTCA	Percutaneous transarterial coronary angioplasty
RACK	Receptors for activated C-kinase
RICK	Receptors for inactivated C-kinase
RNA	Ribonucleic acid
RPM	Revolutions per minute
SAPK	Stress-activated protein kinase
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
SFM	Serum free media
SNP	Sodium nitroprusside
SOD	Superoxide dismutase

SPF	Specific pathogen free
SR	Sarcoplasmic reticulum
STAT	Signal transducer and activator of transcription
SUR	Sulfonylurea receptor
SWOP	Second window of protection
TBS	TRIS-buffered saline
TBST	TBS-Tween
TE	TRIS-EDTA
TEMED	NNNN-tetraethylethalinediamine
TNF	Tumour necrosis factor
TRIS	Tris(hydroxymethyl)methylamine
TTC	Triphenyl tetrazolium chloride
UV	Ultraviolet
UVB	Ultraviolet B
WT	Wild type

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Chapter 1. Ischaemic preconditioning: models, mechanisms and man

1 MYOCARDIAL ISCHAEMIA AND INFARCTION

Myocardial ischaemia can be simply defined as an inadequate blood supply to the heart (1). The blood supply becomes inadequate when the supply of oxygen and nutrients is insufficient to meet the heart's demand. In almost all cases this arises due to a decreased blood flow, which invariably results in an accumulation of metabolites from the ischaemic region, possible contributing to the cellular damage associated with ischaemia.

Myocardial ischaemia usually occurs when one or more of the epicardial coronary arteries becomes narrowed (*stenosed*) or blocked (*occluded*). The consequence to the heart will depend on the degree and, critically, the duration of ischaemia. In some circumstances the degree of ischaemia is mild enough for the heart to function normally at rest, but insufficient to maintain supply during exertion or exercise (*stable angina*). If the depth and breadth of ischaemia is sufficiently great however, the pain associated with angina cannot be relieved by rest (*unstable angina*). Unlike stable angina, which is usually caused by a narrowed coronary artery, unstable angina is probably caused by intermittent blockage of a coronary artery with distal embolization. Often unstable angina steadily worsens (*crescendo angina*) and in a proportion of patients leads to myocardial infarction (*pre-infarction angina*).

Ischaemia can be relieved by a restoration of normal flow, a process known as reperfusion. If reperfusion is not performed soon (20-30 minutes) after a total coronary occlusion, injury will progress from reversible to irreversible injury, when death of cells within the ischaemic risk zone will begin to occur. This process involves coagulative necrosis of the ischaemic tissue and is called acute myocardial infarction.

1.1 The cause of ischaemic heart disease

The underlying cause of ischaemic heart disease is the narrowing of coronary arteries that supply the heart with blood. The constriction of the arterial lumen is brought

about by a thickening of the innermost layer (*intima*) by the development of fibrous tissue and the accumulation of lipid forming atheromatous plaques (*atherosclerosis*). Atherosclerosis is not restricted to the heart, but rather a widespread vascular disease, which is more significant in the heart due to the severe consequences of starving the heart muscle of blood, although atherosclerosis of arteries supplying the brain may be equally severe. Arterial narrowing *per se* is not the cause of major adverse events, but is nonetheless compounded by thrombosis, leading to sudden, total occlusion of the coronary artery and ultimately myocardial infarction. Although the final events leading to thrombolysis are not completely understood, it is believed to be a result of critical changes in plaque status leading to plaque rupture.

1.2 The determinants of cell death

A complete cessation of blood flow to the risk region will result in a profound reaction on myocardial contractility within one minute. The underlying cause is the abolition of oxidative phosphorylation and the consequential decrease in pH and ATP generation. Initially anaerobic glycolysis will maintain homeostasis, however this will only last for a few minutes, during which acidification of the intracellular milieu and build up of extracellular metabolites may only serve to enhance injury. The exact cause of cell death is unknown, but acidification, cell swelling, ATP depletion and calcium overload are all thought to be contribute to the disruption of sarcolemmal integrity and death.

1.3 The extent of the problem

Ischaemic heart disease remains a leading cause of death, accounting for one quarter of all deaths in the UK (2). Although the annual mortality for this disease is decreasing in the UK and other developed countries (3), the figure continues to rise in the more populated developing countries (4). The net effect is that ischaemic heart disease may soon surpass infectious and nutritional diseases as the most common form of death worldwide (4, 5). Despite effective preventative treatments, patients still present with advanced coronary disease with atherosclerotic plaques vulnerable to

events that cause abrupt occlusion, myocardial infarction and death (6). Currently, the most effective treatment is to achieve rapid reperfusion by lysis or mechanical disruption of the occlusive coronary thrombosis and plaque (7, 8). The mortality is inversely related to the amount of myocardial salvage achieved by reperfusion (7), and hence a treatment that slows the rate of myocardial necrosis would invariably increase salvage and decrease mortality (9).

It was a major goal of academic cardiologists, throughout the 1970s and 1980s, to discover a means of protecting the infarcting myocardium (10). Despite a considerable volume of work being published on the subjected, no single agent was found that could consistently and reproducibly limit infarction. It was reasoned that by understanding the biochemical events that lead to irreversible cell injury one might be able to design a strategy to combat ischaemic heart disease. It is ironic that whilst attempting to discover the cause of cell death, Reimer and colleagues serendipitously discovered a method of protecting the myocardium (11), whilst the exact cause of myocardial injury still remains unclear today.

2 INTRODUCTION TO ISCHAEMIC PRECONDITIONING

2.1 The first description

By dissociating ATP depletion and catabolite accumulation, Reimer reasoned that it should be possible to ascertain their relative contributions to ischaemic injury (11). It had been shown in the early 1980s that ATP resynthesis following a single episode of ischaemia was slow, taking as long as four days to recover from a 15-minute coronary occlusion (12, 13). They therefore hypothesised that while ischaemic episodes would induce a cumulative, "stair-step" depletion of ATP, intermittent reperfusions should wash out ischaemic catabolites. Surprisingly their findings showed that, although intermittent reperfusions did cause less cell necrosis, ATP levels were preserved after the first ischaemic episode (11).

In a subsequent study the same group used four 5-minute episodes of ischaemia, interspersed with 5-minute periods of reperfusion, prior to a sustained 40-minute occlusion. Control dogs infarcted 29% of the area at risk, while infarcts where only 7% of the area at risk following brief periods of ischaemia/reperfusion (14). They termed this endogenous protection "preconditioning the myocardium with ischaemia." This phenomenon is now commonly referred to as classical (or early) ischaemic preconditioning.

2.2 Limits of classical preconditioning

The protection against a 40-minute occlusion maybe robust, but this is brought about by a delay in the progression of myocardial necrosis, and hence if the duration of occlusion is increased, the level of protection is diminished. In most species, preconditioning will not protect against an ischaemic time of longer than 90 minutes, and will offer no reduction against permanent occlusion. A possible explanation is that after 60 minutes cell death is mainly occurring in the subepicardium, which can take between 2-5 hours to complete, therefore a delay in this slow progression of injury would not be translated into a large infarct size reduction. Prior to 60 minutes however, the subendocardium is undergoing rapid infarction, which takes only 40 minutes to reach confluency (15).

Unfortunately, the benefit of ischaemic preconditioning is short lived and disappears when the interval between the short ischaemic trigger and the subsequent prolonged ischaemia is greater than 60 minutes (16). In addition, although initially ischaemic preconditioning can be renewed by further brief ischaemic triggers, changes in cell surface receptors and ligand availability, eventually result in tachyphylaxis (17, 18).

2.3 Delayed ischaemic preconditioning

Since the initial discovery of classical preconditioning, a second window has been demonstrated during which the heart is also protected, although to a lesser extent, against a subsequent ischaemic insult (19). This second window of protection

(SWOP) or delayed preconditioning is manifest 12-24 hours after the preconditioning stimulus and lasts for up to 76 hours. The time course of protection appears consistent with a role for gene expression. Many candidate proteins have been proposed as the mediators of delayed preconditioning from inducible nitric oxide synthase (iNOS) (20) to manganese superoxide dismutase (MnSOD) (21). The strongest evidence implicates a member of the heat shock protein (HSP) family, HSP70, since it is consistently elevated 24 hours following preconditioning in most models (22-24). Moreover, induction by heat stress (25) or overexpression (26) of this protein in the absence of preconditioning confers a similar level of protection. It should be noted that there is disagreement with regards the role of HSP70, as in some models protection does not follow HSP induction (27, 28). The mechanisms involved in delayed preconditioning are beyond the scope of this thesis, since they are most likely distinct from those responsible for classical preconditioning.

2.4 A conserved phenomena?

Ischaemic preconditioning has been demonstrated in nearly all species examined including mouse (29), rat (30), rabbit (31), dog (14), and pigs (32). More importantly, preconditioning has been demonstrated in humans using myocardial tissue and the whole heart (see section 5). Furthermore, protection is not restricted to the heart, as it is also seen following brief ischaemia in the kidneys, liver and brain (15).

It is undoubted that harnessing the therapeutic potential of ischaemic preconditioning would save many lives. Unfortunately however, the unpredictability of the moment of coronary occlusion necessitates sustained or renewable protection. These limitations have therefore caused some investigators to question its clinical utility. However, the depth and breadth of the protection afforded by preconditioning are sufficiently impressive to have stimulated and maintained an intense research activity with over 1400 manuscripts published since this phenomenon was described 14 years ago. This interest has focused on the notion that a deeper understanding of the mechanisms underlying preconditioning will allow specific manipulation of the relevant signalling pathways to achieve sustained protection without the risk of an ischaemic trigger.

3 MECHANISMS OF CLASSICAL ISCHAEMIC PRECONDITIONING

The exact mechanisms underlying ischaemic preconditioning remain unknown, however it is not simply caused by an increased blood flow (14), but rather a complex series of intracellular events leading to an adaptive response rendering myocytes resistant to an ischaemic insult. But how does the lack of blood cause the cell to protect itself against further ischaemia? In truth, the answer probably varies between species, but what is common is the requirement for receptor occupancy.

3.1 Triggers of preconditioning

The concept that a blood-borne substance has the capacity to precondition is well accepted. Studies have shown that regional preconditioning in the dog confers protection to a remote area of myocardium that has not been preconditioned (33). Furthermore, either preconditioning of remote organs (34-36) or exposure to coronary effluent from preconditioned hearts (37) conveys protection to the myocardium. If preconditioning can be conveyed in the blood, it is possible that agonists to cell surface receptors are capable of initiating protection.

3.1.1 G-protein coupled receptor agonists

Adenosine was shown to have cardioprotective effects one year prior to the first description of ischaemic preconditioning (38). It was also known that adenosine is rapidly produced and released by the heart during brief coronary occlusion, as ATP catabolism exceeds synthesis (39). It is not surprising therefore that adenosine was the first heptahelical transmembrane receptor agonist implicated in ischaemic preconditioning (40)

3.1.1.1 Adenosine

Using an open-chest rabbit model, Downey's group originally tested the hypothesis that preconditioning is triggered by the activation of cardiac adenosine receptors. They demonstrated that adenosine was both necessary and sufficient for protection, since antagonists abolished and exogenous adenosine mimicked the protective effect of preconditioning (40). Later the same group implicated the A_1 receptor subtype by the use of subtype specific agonists (41), although activation of the A_3 receptor, which was not examined by Downey's group, has been shown to protect in the rabbit (42). The concentration of adenosine may determine the profile of protection because agents that augment tissue adenosine levels lower the threshold (43), potentiate (44) and extend the window (45) of ischaemic preconditioning. Substantial evidence now suggests adenosine receptor activation evokes a preconditioning response, except perhaps in the rat were adenosine may not protect (46-48).

Adenosine is released, not only during preconditioning, but also during the index ischaemia. This release is reportedly enhanced in preconditioned myocardium, possibly due to the increased activity of 5'-nucleotidase (49, 50). The activation of adenosine receptors during index ischaemia is also responsible for protection in response to preconditioning (51, 52). This would suggest that adenosine is not only a trigger but also a mediator of protection. The most likely explanation is that adenosine receptor activation during preconditioning acutely upregulates adenosine release during lethal ischaemia, which contributes to protection.

Adenosine activates intracellular signalling pathways through heterotrimeric GTP-binding proteins (G-proteins). These intracellular pathways maybe conserved between different G-protein coupled receptors (GPCRs), since other GPCR agonists can similarly protect the myocardium against ischaemia.

3.1.1.2 Others

Other GPCRs implicated in preconditioning include bradykinin (53), endothelin-1 (54), α_1-adrenergic (55), opioid (56) and angiotensin II receptors (57). None of these receptors are ubiquitously responsible for protection, but their relative contributions are both species- and model-dependent. For example, although blockade of either adenosine (58, 59) or α_1-adrenergic (55) receptors is sufficient to abolish preconditioning in rabbit heart, simultaneous inhibition of both receptors in isolated rat heart does not prevent protection (60). Furthermore, although HOE 140 (bradykinin B_2 receptor blocker) abolished protection by 5 minutes ischaemia and 10 minutes reperfusion in isolated rabbit hearts, when the preconditioning stimulus was amplified by four cycles of ischaemia/reperfusion HOE 140 did not alter infarct size (53). On the basis of these results, Goto and co-workers proposed that GPCRs couple to a unique substrate responsible for protection, and the blockade of either of these receptors could diminish the stimulus below a threshold and thus prevent protection. A more intense preconditioning stimulus can overcome specific receptor blockade by the potentiated agonism of other receptors (Figure 1-1). Goto and co-workers proposed that protein kinase C (PKC) was the unique substrate on which these initial signals converge to cause protection (53).

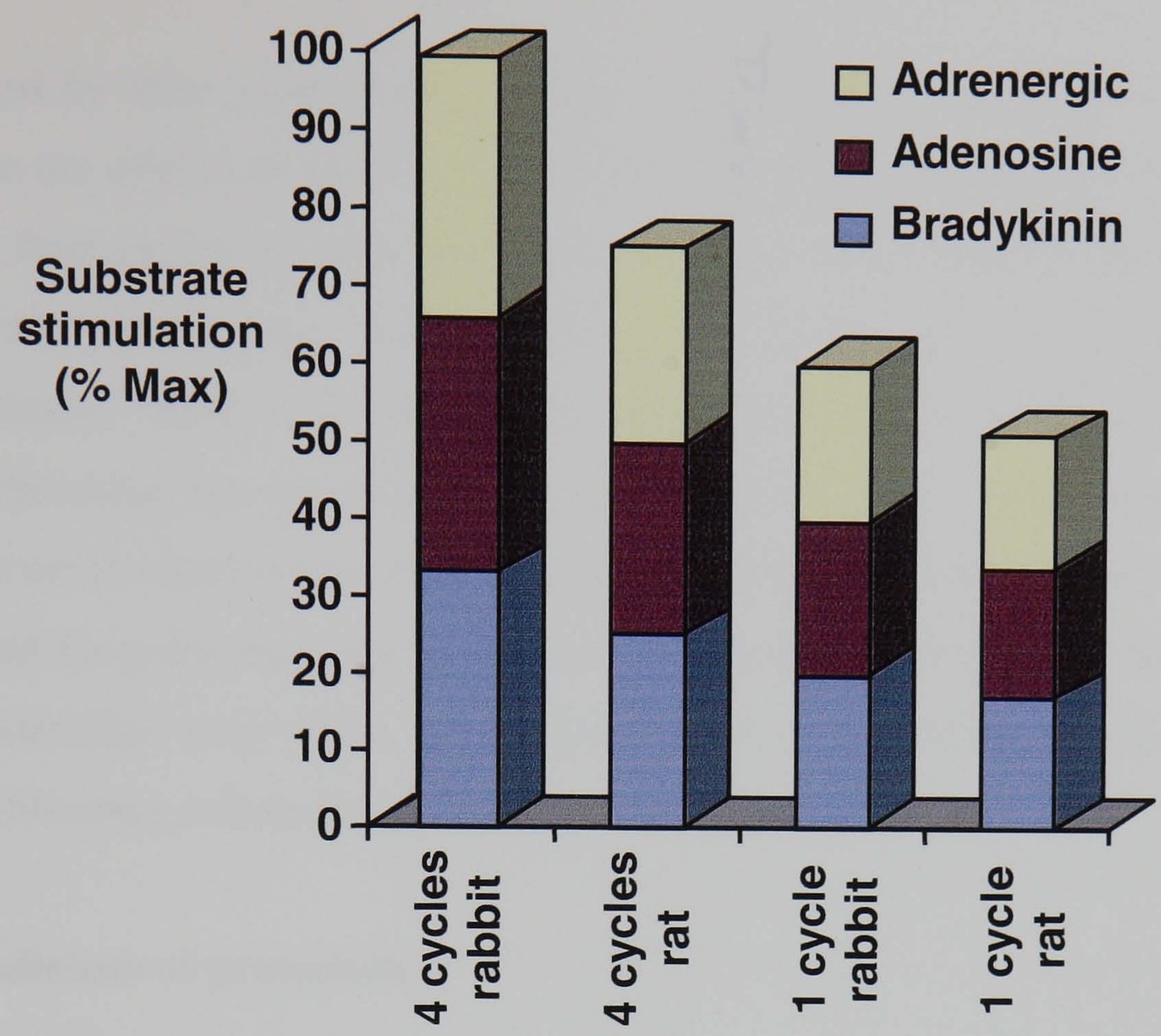

Figure 1-1: Schematic representation of the threshold theory for preconditioning. If the common downstream substrate needs to be stimulated by 50% to produce an effect, then antagonism of either receptor during preconditioning with 1 cycle of ischaemia/reperfusion would abolish protection in rabbit heart. However, after 4 cycles of ischaemia/reperfusion in the rabbit, combined receptor antagonism would be necessary to block protection.

3.1.2 Free radicals

Free radicals are released during reperfusion following ischaemia (61), and possibly during ischaemia itself (62). Previous studies have shown that low levels of free radicals can activate PKC directly (63) or indirectly via phospholipase D (PLD) activation and subsequent diacylglycerol (DAG) production (64). It is possible therefore that free radical generation during preconditioning may trigger protection by mechanisms similar to those initiated by GPCR activation. Unfortunately investigations addressing this hypothesis have produced conflicting data. In the rabbit, Tanaka *et al.* reported that protection following a single episode of preconditioning was completely abolished by the free radical scavengers superoxide dismutase (SOD) or N-2-mercaptopropionylglycine (MPG) (65). In a similar model, but using four preconditioning cycles, Iwamoto *et al.* failed to block the anti-infarct effect by scavenging free radicals (66). These apparently contradictory findings may be

explained by later work from Downey's group who used a similar rabbit model to examine the effects of MPG on different preconditioning protocols (67). Using either one or four cycles of preconditioning ischaemia and reperfusion they observed a significant infarct size reduction after 30 minutes regional ischaemia *in vivo*. Interestingly, MPG was able to abolish protection resulting from one cycle of preconditioning, but not in response to four cycles. The authors concluded that free radicals act in concert with other activators of PKC (i.e. GPCR agonists) to achieve a threshold for protection, and that free radical inhibition would only abolish protection if the stimulus were close to the threshold for preconditioning (i.e. one cycle of preconditioning ischaemia).

3.2 Mediators of protection

3.2.1 Heterotrimeric GTP-binding proteins (G-proteins)

Upon ligand binding, the α-helical membrane spanning regions of GPCRs undergo a profound change, which affects the conformation of the intracellular loops and uncovers previously masked G-protein binding sites (68). The subsequent interaction with heterotrimeric G-proteins promotes the release of bound guanosine diphosphate (GDP) from the α subunit and its exchange for guanosine triphosphate (GTP). This in turn stimulates the dissociation of the α subunit from the βγ dimer (68). The activated G-protein subunits then initiate intracellular signalling responses by acting on a variety of effector molecules.

Four families of G-protein α subunits have been identified; G_s, G_i, G_q, and G_{12}. The subunits involved in preconditioning are $(G_i)/G_q$ since selective inhibition of these during preconditioning with pertussis toxin is sufficient to abolish protection (69, 70). The most likely targets for these subunits are phosphatidylinositol-specific phospholipases, in particular phospholipase C (PLC).

3.2.2 Phospholipase C/D

Phospholipase C, a downstream substrate for G_q subunits, hydrolyses phosphatidylinositol 4,5-biphosphate (PIP_2) to inositol 1,4,5-triphosphate (IP_3) and diacylglycerol (DAG) (71). IP_3 and DAG in turn lead to the activation of a number of protein kinases, including protein kinase C (72). This indirect evidence suggests PLC links GPCRs, such as adenosine and bradykinin, to intracellular kinases, such as PKC (73). More direct evidence however, favours the involvement of phospholipase D (PLD). During preconditioning the activity of PLD is enhanced 2-fold and specific activation in the absence of preconditioning is sufficient to protect (74). PLD can similarly activate PKC via the production of DAG, although whether it is regulated by G-proteins is controversial, as intracellular calcium levels and tyrosine phosphorylation are both thought to contribute to PLD activation (75).

3.2.3 Protein kinase C

3.2.3.1 The protein kinase C family

3.2.3.1.1 Classification

Protein kinase C (PKC) comprises a ubiquitous family of Ser/Thr kinases that play a critical role in many intracellular signal-transducing pathways. Members of the PKC family exhibit diversity in an N-terminal region, termed the regulatory domain. Structural differences within this domain confer variations in cofactor requirement, which divide the PKC family into three subfamilies. The conventional subfamily (cPKC: α, β_1, β_{11} and γ) is dependent upon both Ca^{2+} and diacylglycerol (DAG) for activation, the novel subfamily (nPKC: δ, ε, η and θ) is Ca^{2+} independent but DAG dependent, and the atypical subfamily (aPKC: ζ and ι) is both Ca^{2+} and DAG independent (see Figure 1-2).

3.2.3.1.2 Structure

The primary structure of PKC comprises an N-terminal regulatory region and a C-terminal catalytic domain, separated by a hinge region. The N-terminal region contains two conserved domains, termed C1 and C2, which constitute the membrane-targeting regulatory domains that are required for interaction with Ca^{2+}, phosphatidylserine (PS), DAG and phorbol esters. The classification of PKC isoforms is entirely based on the structure of these conserved domains (see section 3.2.3.1.1 and Figure 1-2). Although PKC isozymes are grouped into subfamilies, each individual isoform has up to five unique variable regions (V1-V5). Although some of these variable regions affect substrate specificity (76, 77), most mediate protein-protein interactions, thereby enabling differential localisation of PKC isozymes within the cell (78-81). Through a combination of localisation, activation and perhaps substrate specificity, individual PKC isozymes can cause distinct physiological effects. For example, members of the same sub-family, PKCδ and ε, have been shown to produce opposing effects on growth and morphology (82).

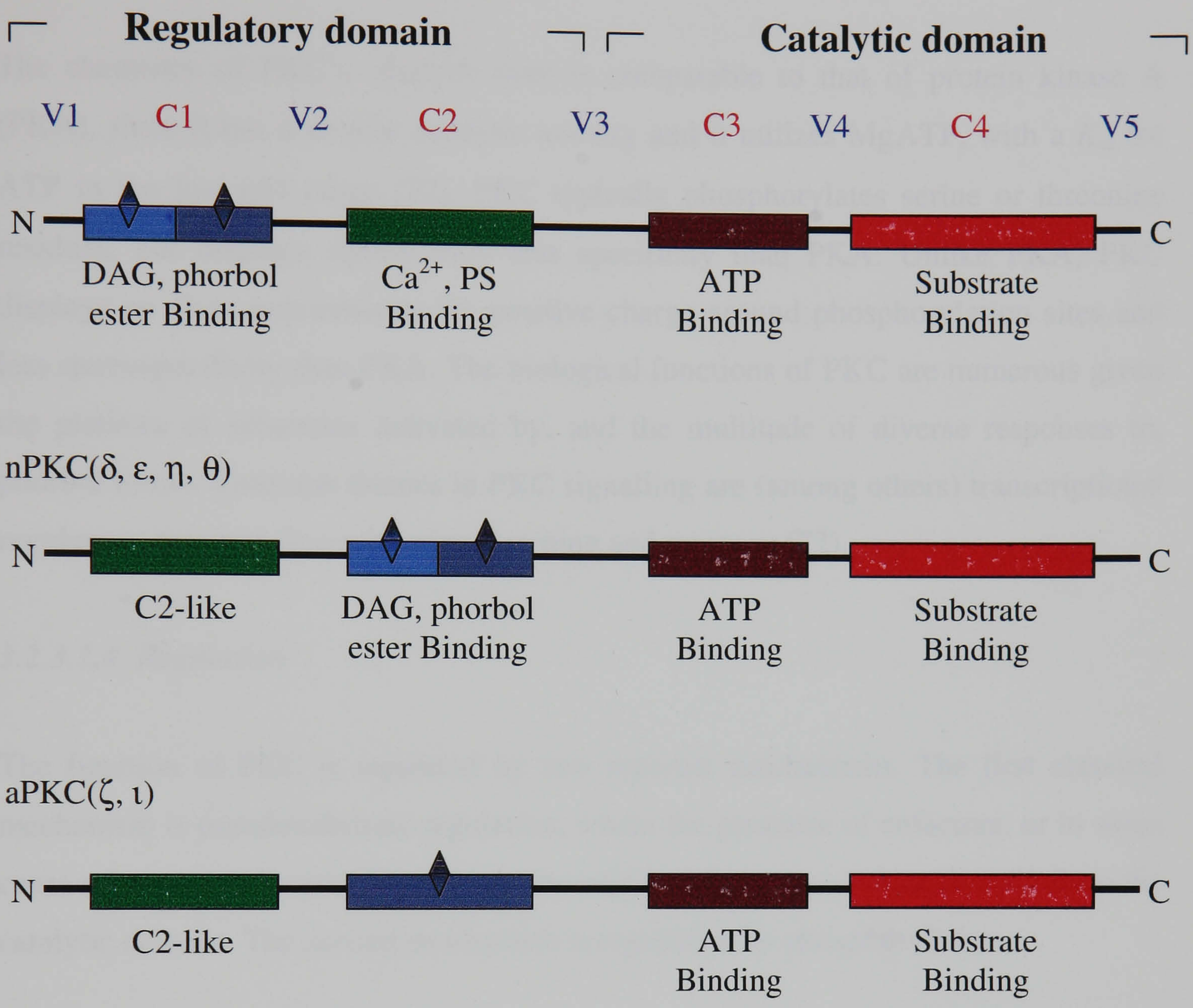

Figure 1-2: The sequence homology between the primary structures of protein kinase C (PKC) isoforms.
Members of the PKC family are classified according to the presence of four conserved domains (C1-C4). Whilst all members have a C3 and C4 domain in the catalytic region, responsible for binding ATP and PKC substrates respectively, the C1 and C2 domains within the regulatory region are more diverse. The classical C1 domain possesses two cystein-rich zinc fingers which co-ordinate the binding of diacylglycerol (DAG) and phorbol esters, whereas the C2 domain binds Ca^{2+} and phosphatidylserine (PS). PKC isoforms containing all these conserved regions are termed *classical* PKCs (cPKC). The *novel* PKC (nPKC) and *atypical* PKC (aPKC) groups both posses a "C2-like" domain, and the aPKC group only possess one zing-finger domain, rendering it resistant to DAG and phorbol ester binding. Diversity within groups is achieved through five variable regions (V1-5), which mediate protein-protein interactions (Adapted from Newton *et al.* (72)).

3.2.3.1.3 Function

The chemistry of PKC's catalytic core is comparable to that of protein kinase A (PKA), since it has a similar catalytic activity and it utilizes MgATP, with a K_m for ATP in the low μM range (72). PKC typically phosphorylates serine or threonine residues, but displays significantly less specificity than PKA. Unlike PKA, PKC displays no clear requirements for positive charge around phosphorylation sites and less stereospecificity than PKA. The biological functions of PKC are numerous given the plethora of substrates activated by, and the multitude of diverse responses to, phorbol esters. Common themes in PKC signalling are (among others) transcriptional regulation, receptor desensitisation, learning and memory (72).

3.2.3.1.4 Regulation

The function of PKC is regulated by two separate mechanisms. The first classical mechanism is pseudosubstrate regulation, where the presence of cofactors, or in some cases substrates, is required to remove the inhibitory pseudosubstrate domain from the catalytic domain. The second mechanism is regulation by phosphorylation.

3.2.3.1.5 Pseudosubstrate regulation

The extreme N-terminal portion of PKC contains a short stretch of amino acids that have a strong affinity for the catalytic C4 domain. This short sequence of amino acids, which prevents substrate phosphorylation by binding to the catalytic domain itself, is termed the pseudosubstrate domain. PKC is activated by the removal of the pseudosubstrate region from the kinase core. This is typically brought about by a conformational change in PKC by binding, through the regulatory domain, to lipid membranes. Conventional activators of PKC, such as phorbol esters and DAG, work by anchoring PKC to the membrane by interaction with the C1 domain. The regulation of PKC activity by the pseudosubstrate domain is therefore dependent on tertiary structure, which is in turn determined by cofactors.

The pseudosubstrate regulation of PKC complicates the measurement of PKC activity *in vitro* because kinase assays must be performed in the presence of DAG and PS, which makes extrapolations to cellular levels of activity imperfect. These and other technical limitations in the direct intracellular measurement of PKC isoform activity have prompted investigators to use translocation as a surrogate for PKC activation (83). This is based on the premise that cytosolic PKC is inactive, whereas membrane bound PKC is active, thus measurement of the movement of PKC between the cytosol and membrane will give an indication of activity.

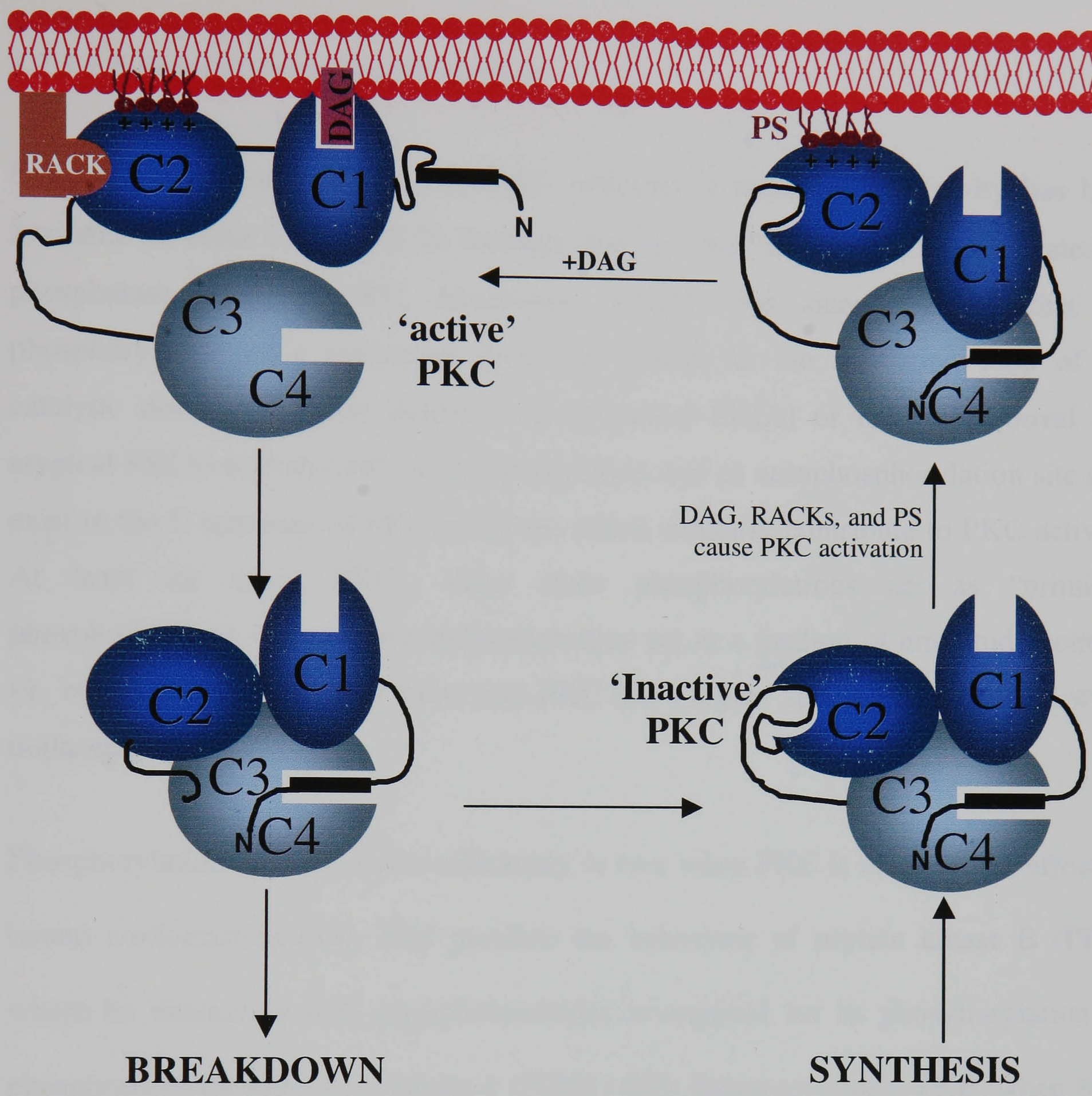

Figure 1-3: Diagrammatical representation of PKC activation and degradation.
PKC contains four conserved domains. The C1 and C2 domain, which make up the regulatory region, bind diacylglycerol (DAG) and phosphatidylserine (PS) respectively. The C3 and C4 domains, which are contained within the catalytic region, contain key residues responsible for ATP binding and downstream substrate phosphorylation. In the inactive state, PKC resides in the cytosol and an inhibitory pseudosubstrate region prevents substrate phosphorylation by binding to the C4 domain and blocking the kinase core. Upon binding of DAG to the C1 domain, and calcium to the C2 domain (for classical PKCs), the affinity of PKC for PS and is greatly increased (84). The binding of PS to the C2 domain causes a conformational change resulting in removal of the pseudosubstrate region from the catalytic site and exposure of the receptor for activated C-kinase (RACK) binding site. The net result is the localisation of 'active' PKC to its respective RACK and the phosphorylation of localised downstream substrates. Adapted from Newton *et al.* (84).

3.2.3.1.6 Regulation by phosphorylation

Evidence that phosphorylation of PKC isoforms is required for activity has been available for some time, with the findings that purified PKCα could be inactivated by phosphatase treatment (85). Mutational analysis has since revealed that the phosphorylation of a conserved threonine residue in the activation loop of the catalytic domain is either essential for (classical PKCs) or enhances (novel and atypical PKCs) activity (86). A phosphorylation and an autophosphorylation site also exist in the C-terminus of PKC isoforms, which similarly contribute to PKC activity. At least for novel PKCs, these three phosphorylations act as "priming" phosphorylations, because in combination they act as a method of amplitude control, i.e. when phosphorylated in these sites PKC has a higher specific activity than when unphosphorylated.

Phosphorylation of PKC occurs efficiently *in vivo* when PKC is in an active, effector-bound conformation (86). This parallels the behaviour of protein kinase B (PKB) where its interaction with phosphoinositides is required for its phosphorylation by phosphoinositide-dependent kinase-1 (PDK1) (87). However, unlike PKB, when PKC releases its activator (DAG) its phosphorylations are not rapidly lost. In fact, this closed conformation of PKC appears relatively resistant to phosphatases, which enables PKC to remain phosphorylated for tens of minutes to hours. The individual kinases involved in PKC phosphorylation are outline in Figure 1-4.

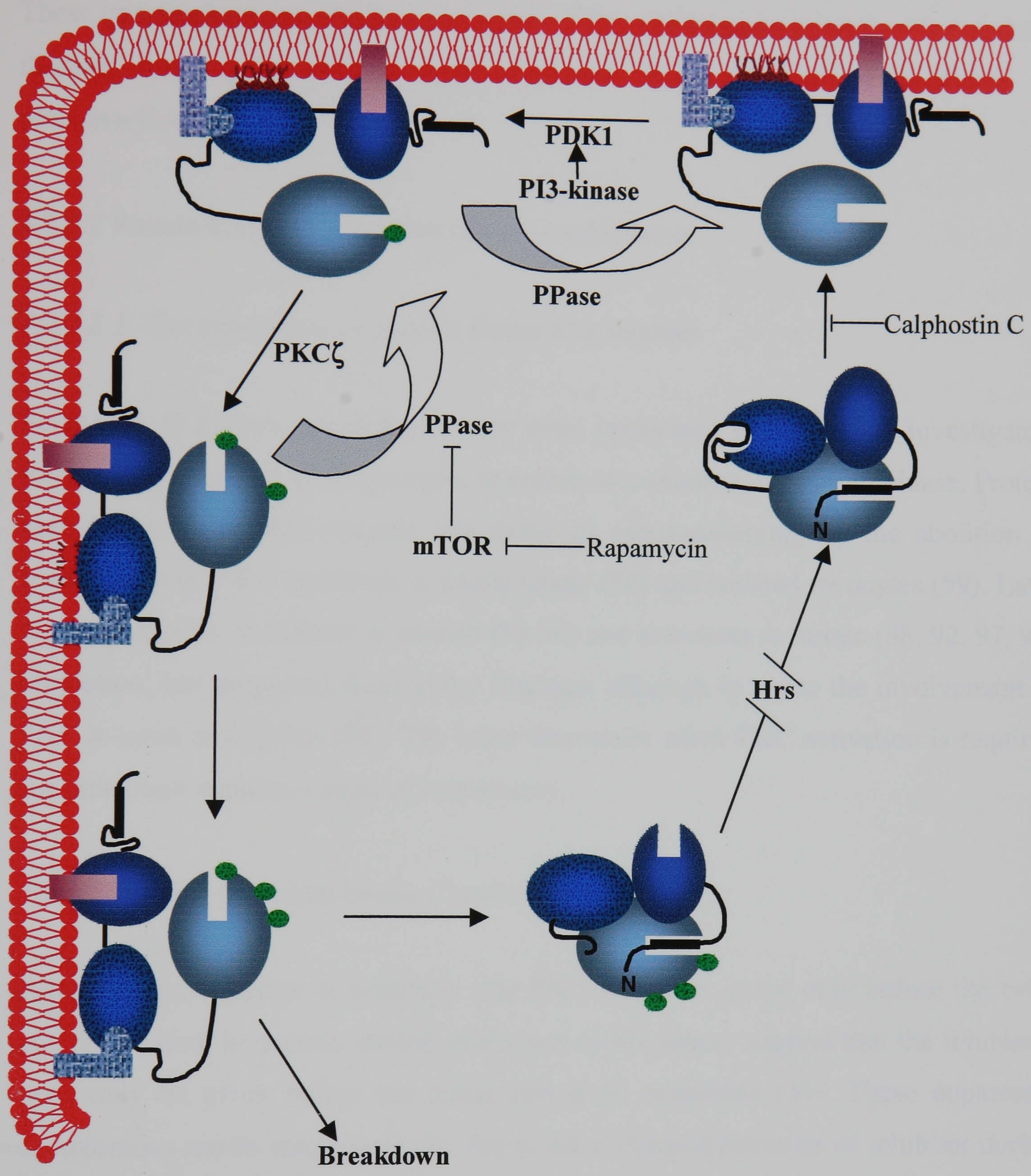

Figure 1-4: Schematical representation of the three 'priming' phosphorylations that increase PKC activity.
In the presence of cofactors, such as PS and DAG, PKC translocates from cytosol to membrane. This translocation can be blocked by inhibitors that bind to the regulatory domain, such as calphostin C. Upon membrane binding, PKC becomes susceptible to phosphorylation (green circles) on a threonine residue within the activation loop. The co-recruitment of phosphoinositide-dependent kinase-1 (PDK1) to the membrane probably enables this enzyme to catalyse the phosphorylation of this threonine residue within the activation loop. After a further phosphorylation by PKCζ and a subsequent autophosphorylation, PKC is either targeted for dephosphorylation (and degradation) or released from the membrane in its closed catalytically competent form into the cytosol. In this state PKC is relatively resistant to dephosphorylation and can remain in this "primed" conformation for many hours. If PKC is activated within this time by membrane recruitment, its specific activity will be much greater than when unphosphorylated.

These two mechanisms of regulation enable PKC activity to be "switched" on and off, through the requirement for lipid cofactors, and modulated in amplitude by phosphorylation.

3.2.3.2 Protein kinase C and ischaemic preconditioning

3.2.3.2.1 The importance of protein kinase C activation

Soon after G-protein coupled receptors were implicated in protection, investigators focussed downstream of the receptor in search of a common signalling kinase. Protein kinase C was the first enzyme implicated in preconditioning, by the abolition of protection with PKC inhibitors in whole hearts (88) and isolated myocytes (59). Later work, using PKC inhibitors to abolish (89-96) and activators to mimic (88, 92, 97, 98) protection, has supported these initial findings, although in swine the involvement of PKC is more ambiguous (99). The exact time-point when PKC activation is required for protection remains a topic of controversy.

3.2.3.2.2 When is protein kinase C activation important?

Although some groups demonstrate that PKC inhibitors given only before the brief preconditioning ischaemia abolish protection (100), others suggest that the inhibitors need only be given before the lethal period of ischaemia (94). These apparently dichotomous results may simply be due to the continued presence of inhibitor during lethal ischaemia either through incomplete washout or the use of *in vivo* models with a closed circulation. Downey and colleagues showed that PKC activity was required during index ischaemia, since the catalytic domain inhibitor staurosporine blocked protection only when give before the onset of lethal ischaemia (101). They also showed however that PKC translocates to the membrane during preconditioning and colchicine, which disrupts cytoskeletal microtubules and presumably PKC translocation, blocks protection during preconditioning. Downey therefore hypothesised that PKC translocation is required during preconditioning, whereas its activity was necessary during lethal ischaemia.

Whilst most PKC inhibitors work by binding to the PKC catalytic domain, others bind to the regulatory domain. The catalytic domain of PKC has a high degree of sequence homology with other protein kinases, hence most PKC inhibitors also inhibit other protein kinases. However, calphostin C and chelerythrine are thought to bind to the regulatory domain since they do not affect catalytic activity *in vitro* (P Cohen, personal communication). These inhibitors do however prevent preconditioning and cause an altered pattern of PKC distribution on fractionation (102, 103). Furthermore, although ischaemic preconditioning has no effect on total PKC activity in rabbit myocardium, protection is abolished by chelerythrine, which alters the fractionation pattern of PKCε and τ (88). This requirement for PKC translocation has recently been exploited to investigate the individual PKC isoforms responsible for protection.

3.2.3.2.3 The specific involvement of protein kinase C isoforms

The high sequence homology within the catalytic domain of different PKC isoforms complicates the development of isoform-specific inhibitors that block catalytic activity. In contrast, variable regions within the regulatory domain, which dictate spatial localisation and therefore downstream substrate activation, represent a viable target for isoform-specific inhibition. Based on this hypothesis, Mochly-Rosen and co-workers discovered unique sequences that co-ordinate binding to discreet intracellular receptors (104). These intracellular receptors for activated C-kinase (or RACKs) bind specific PKC isoforms in their active conformation and localise them with their downstream substrates. The model for the interaction of PKC with RACKs (adapted from (105)) is shown in Figure 1-5.

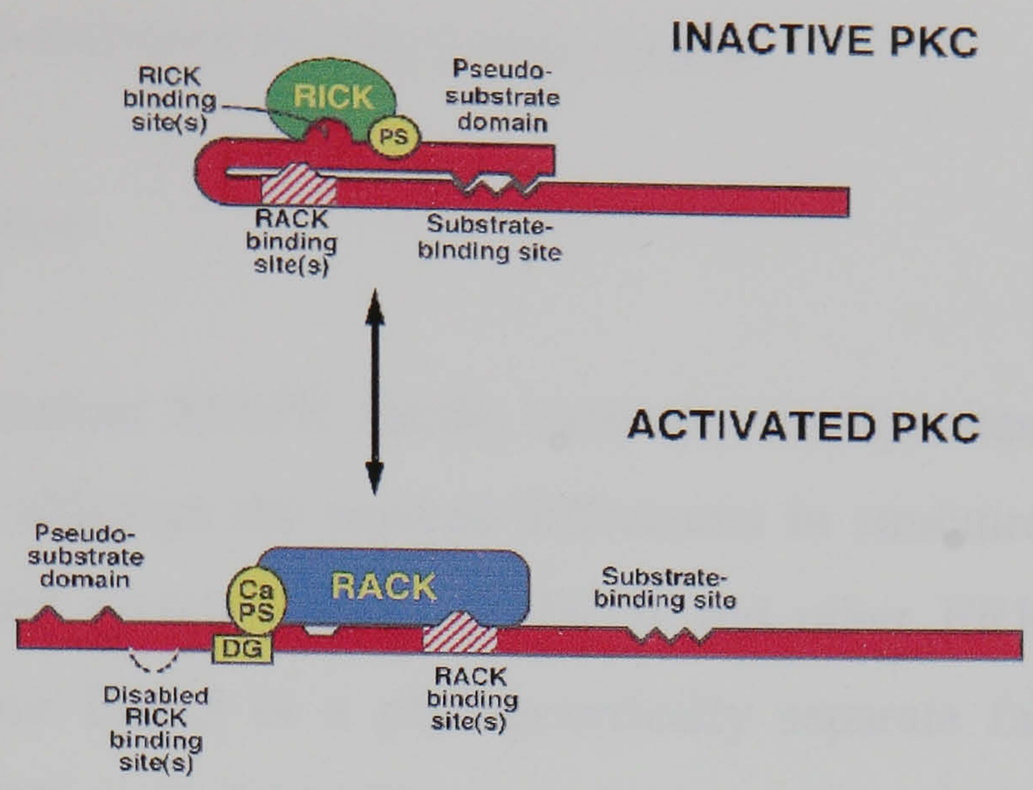

Figure 1-5: Model of interaction between receptors for active C-kinase (RACKs) and PKC.
When inactive, PKC is in a closed conformation, which prevents substrate phosphorylation. Upon binding of diacylglycerol (DAG) and phosphatidylserine (PS) the RACK binding site becomes accessible which promotes translocation to, and binding of, the respective isoform-specific RACK. This stabilises PKC in the active conformation and localise it with its downstream substrates (adapted from Mochly-Rosen *et al.* (105)).

By introducing peptides, which correspond to the RACK sequence on PKC isoforms, into isolated myocytes, Mochly-Rosen's group were able to prevent the translocation of individual PKC isoforms (106). Using this approach during preconditioning, they were able to show that PKCε translocation was necessary for subsequent protection (107, 108). Moreover, protection can be mimicked by peptides that cause PKCε translocation to its respective RACK (109).

3.2.4 Mitogen-activated Protein Kinases

It was in 1988 that Ray and Sturgill noticed that a 42KDa protein, upon tyrosine and threonine phosphorylation, became a serine-threonine kinase (110). The sensitivity of this protein to activation by mitogens, such as PMA, resulted in the term mitogen-activated protein kinase (MAPK). Later however, after the realisation that it could be stimulated by a diverse variety of extracellular signals, the term extracellular signal-regulated kinase (ERK) was adopted.

3.2.4.1 The Mitogen-activated Protein Kinase family

3.2.4.1.1 Classification

The classical mammalian MAPK family currently incorporates at least 5 different proteins (ERK1-5), although the marked differences in structure between ERK5 (or big mitogen-activated protein kinase [BMK1]) and other ERKs has caused some investigators to place ERK5 in a phylogenetically separate family. Following the discovery of the ERKs, other MAPKs have been identified which are activated by cellular stresses such as UV radiation and hyperosmotic shock. This group of MAPKs, termed stress-activated protein kinases (SAPKs), can be further subdivided into c-Jun N-terminal kinases (or JNKs) and p38-MAPKs (Figure 1-6).

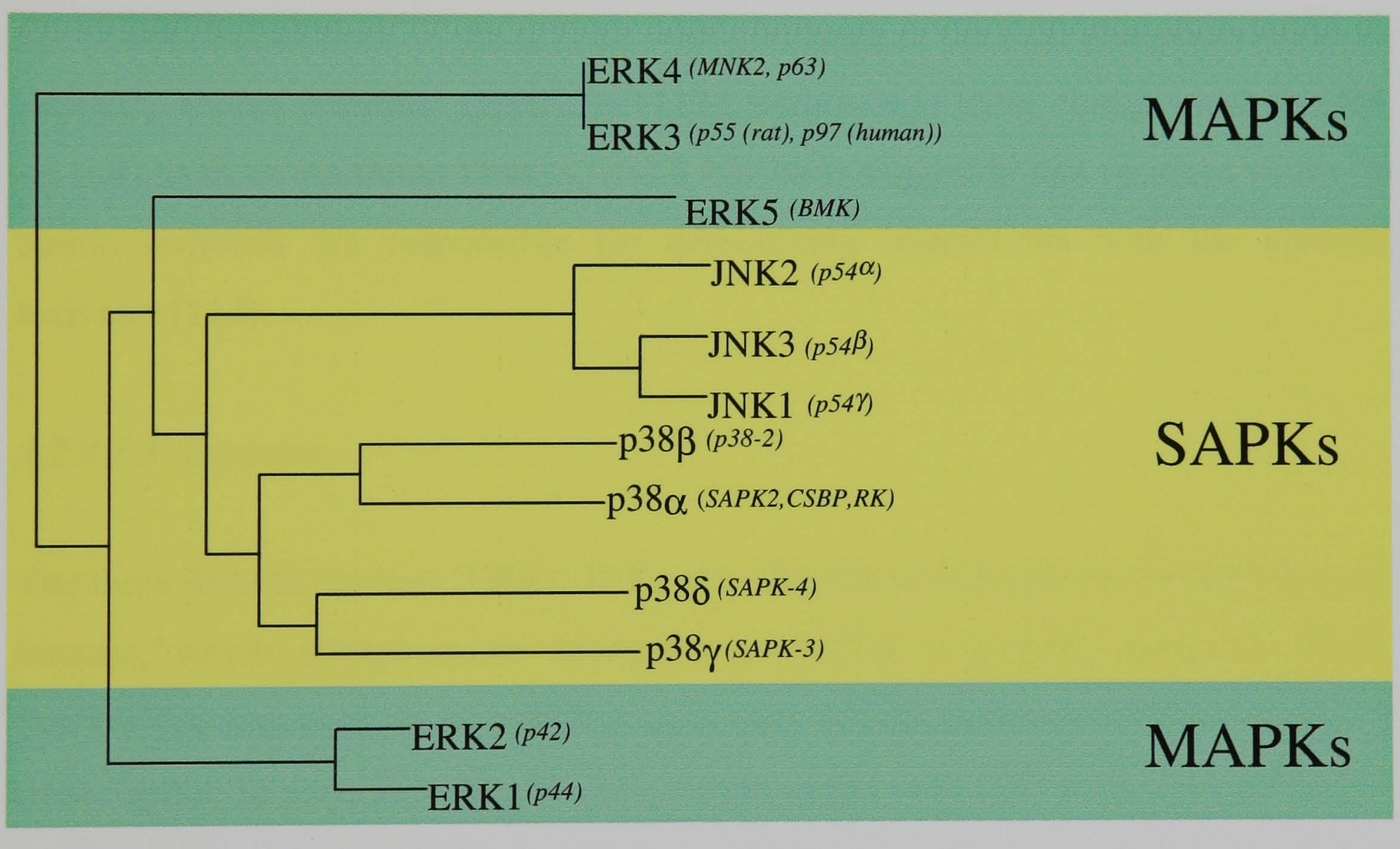

Figure 1-6: Phylogenetic tree of mammalian mitogen-activated protein kinase (MAPK) family members.
Dendrogram was adapted from Zhao *et al.* (111), were they were created, using human sequences when available or mouse sequences otherwise, with CLUSTAL X program.

3.2.4.1.2 Structure

In a manner analogous to many other protein kinases, MAPK activity is regulated by phosphorylation. The key difference however, is the essential requirement for dual

phosphorylation within a canonical amino acid sequence in the activation loop lying between subdomains VII and VIII (111). This is achieved by dual specificity MAPK Kinases (MAPKKs or MKKs) that recognizes and phosphorylate a Thr-Xxx-Tyr motif in the activation loop. For each of the three MAPK families the "Xxx" residue within this targeting sequence differs, glutamate for the ERKs, proline for JNKs and glycine for the p38s. Following dual phosphorylation by upstream MKKs, the activation loop refolds and interacts with surface arginine-binding sites that stabilise the altered conformation of the loop together with neighbouring sequences, resulting in activation of the kinase (112). The activation loop itself has been shown to confer differences in substrate recognition between different MAPK family members. For example, changes in the amino acid sequence within the loop of p38 to a sequence that resembles ERK shifts the selectivity of downstream substrates from p38 to ERK. Moreover, replacement of the glycine with glutamate in the dual phosphorylation site similarly altered substrate specificity (113). Surprisingly these changes had no effect on selectivity to upstream kinases, and it has been suggested that residues within the amino terminus are responsible for determining interactions with the upstream activator (114).

3.2.4.1.3 Function

The three MAPK families (ERKs, JNKs and p38) are proline directed serine/threonine kinases, which phosphorylate substrates containing a general consensus Pro-X-Ser/Thr-Pro sequence. In mammalian cells, most MAPK substrates defined to date are transcription factors, including ELK-1, c-myc, c-jun, and ATF-2 (see Figure 1-7). In addition, several cytoskeletal proteins, protein kinases and phospholipases are substrates for specific MAPKs. As our understanding of these pathways increase, it is likely that additional classes of substrates will be defined. The physiological consequences of MAPK activation vary dramatically between different MAPKs, different isoforms of the same kinase, and different cell types. For example, p38-MAPKs and ERKs have opposing roles on cell survival, with p38 promoting apoptosis and ERK1/2 survival (115). A similar dichotomy has been shown in neonatal cardiac myocytes regarding the contribution of p38α and β to apoptosis and

survival, respectively (116). Moreover, the same MAPK can cause opposing effects dependent on cell type, as p38 activation leads to apoptosis in PC12 cells (117), but promotes survival in BaF3 cells (118). In mammalian systems, the activation of these protein kinases can lead to a diverse array of regulatory events including differentiation, proliferation, development, apoptosis and inflammation.

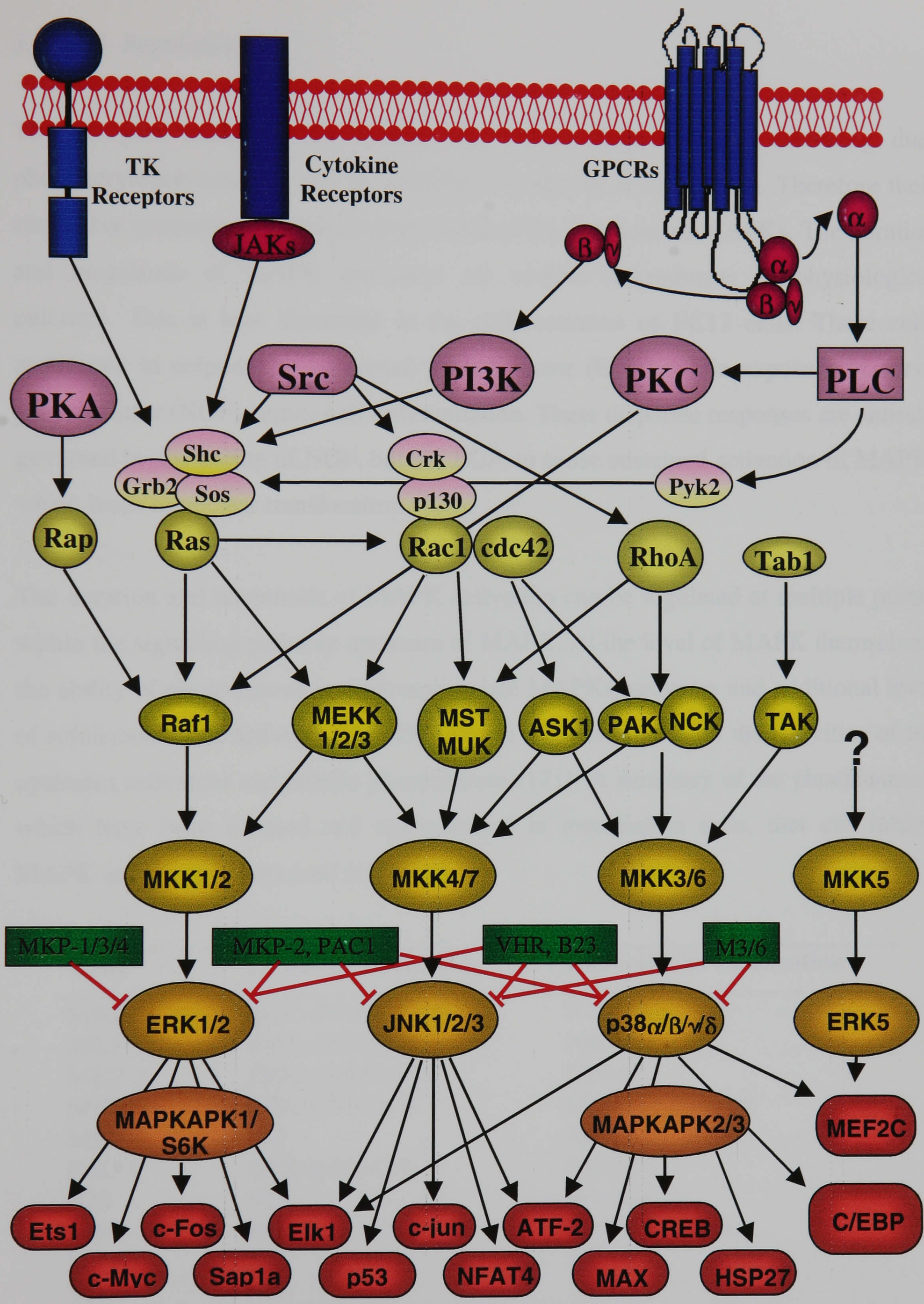

Figure 1-7: Representation of the intracellular signalling pathways that can activate, and are activated by, MAPK cascades.

3.2.4.1.4 Regulation

The complex intramolecular change that occurs within MAPK following dual phosphorylation causes a 100 to 1000-fold increase in activity (119). Therefore their respective upstream activators acutely regulate the functions of MAPKs. The duration and magnitude of MAPK activation are critical determinants of physiological outcome. This is best illustrated in the differentiation of PC12 cells. These cells proliferate in response to epidermal growth factor (EGF), while exposure to nerve growth factor (NGF) causes cell differentiation. These disparate responses are entirely governed by the ability of NGF, but not EGF, to cause sustained activation of MAPK, which leads to nuclear translocation (120).

The duration and magnitude of MAPK activation can be regulated at multiple points within the signalling pathway upstream of MAPK. At the level of MAPK themselves, the ability of phosphatases to dephosphorylate MAPKs, provides and additional level of refinement. The activity of MAPK reflects a balance between the activities of the upstream activators and protein phosphatases (121). A summary of the phosphatases, which have been isolated and characterised in mammalian cells, that can inhibit MAPK activity are illustrated in Table 1-1.

Gene	Substrate Specificity	Intracellular localisation
MKP-1	ERK=JNK=p38	Nucleus
MKP-2	ERK=JNK>p38	Nucleus
MKP-3	ERK>>JNK=p38	Cytosol
MKP-4	ERK>JNK>p38	Cytosol> nucleus
MKP-5	ND	ND
PAC-1	ERK=p38>JNK	Nucleus
B23	ND	Nucleus
M3/6	JNK=p38>>ERK	Cytosol, nucleus
MKPX	ND	ND
VHR	ND	ND

Table 1-1: The characterisation of members of the mammalian dual-specificity MAPK phosphatase (MKP) family.

3.2.4.2 Mitogen-activated protein kinases in ischaemic preconditioning

In 1996, MAPK activation was demonstrated during ischaemia/reperfusion in the perfused heart (122) and during hypoxia/re-oxygenation in isolated myocytes (123). Shortly afterwards Maulik *et al.* reported an increase in MAPK activity, and in particular MAPKAPK2, a downstream substrate of p38, following preconditioning in isolated working rat hearts (124). Since this initial manuscript the involvement of MAPK in preconditioning has been a subject of intense investigation. Unfortunately however, almost five years later, the relative contributions of MAPK isoforms to ischaemic preconditioning remain controversial, with contradictory reports suggesting that MAPK activation is good during preconditioning, bad during ischaemia and not associated with protection or injury (125).

3.2.4.2.1 The good, the bad and the ugly

The first clear evidence of MAPK phosphorylation in response to preconditioning came from Downey's group. In particular, the phosphorylation of p38-MAPK on tyrosine 182 was enhanced during ischaemia following preconditioning. Moreover, this phosphorylation was abolished if protection was blocked pharmacologically (126). As discussed earlier, dual phosphorylation of both tyrosine and threonine residues is necessary for MAPK activation, and unfortunately phosphorylation of the neighbouring threonine residue or total p38 activity was not measured. In a parallel study using isolated adult myocytes rendered ischaemic by pelleting under mineral oil, inhibition of p38 with SB203580 abolished the resistance to osmotic shock following preconditioning (126). Furthermore, preconditioning was mimicked by the SAPK activator anisomycin. More recently, the same group have confirmed these findings in the isolated perfused rabbit heart by measuring activity of mitogen-activated protein kinase activated protein kinase-2 (MAPKAPK-2), a downstream substrate of p38 (127). In heart biopsies, a fraction was eluted that was able to phosphorylate a peptide sequence that is a known substrate for MAPKAPK-2. This activity was present during prolonged ischaemia in preconditioned but not control hearts (127). Once again there was no direct measurement of p38-MAPK activation or

dual phosphorylation, although MAPKAPK-2 activities correlated with protection, even when pharmacological manipulations were used to initiate or prevent preconditioning. Other groups have also shown that p38 dual phosphorylation (128) and MAPKAPK2 activities (124, 129) are enhanced in preconditioned hearts compared to controls, and that inhibition of p38 activity with SB203580 abolishes protection (128, 130). *Taken together these data suggest that p38-MAPK activation following preconditioning is causally related to protection.*

Unfortunately this body of data contradicts the findings of others with regard to the role of p38-MAPK during ischaemia. For example, p38-MAPK is phosphorylated during ischaemia/reperfusion and inhibition of this activation with SB203580 is protective in isolated cells (131) and the whole heart *in vitro* (132) and *in vivo* (133). Furthermore, prolonging and enhancing p38 activation with a tyrosine phosphatase inhibitor aggravates ischaemic injury (134). A similar relationship between p38 and injury appears to exist in human myocardium, because inhibition of p38-MAPK during simulated ischaemia and re-oxygenation in isolated human atrial trabeculae improved post ischaemic functional recovery (135). The authors concluded that TNFα release was responsible for injury in response to p38 activation during ischaemia, since this was inhibited with SB203580, and TNFα suppressed myocardial contractility when given alone (136). These findings may have clinical relevance since p38 activation has been detected in the human heart during coronary artery bypass surgery (137). *Taken together these data suggest p38 activation during ischaemia contributes to myocardial injury and/or contractile depression.*

These findings are further clouded by a recent study by Behrends *et al.*, which measures ERK1/2, JNK1/2 and p38 activation during ischaemia in anaesthetised pigs (138). By taking biopsies, they were able to measure activation in hearts subjected to ischaemia and/or preconditioning. The variable level of MAPK activation seen during ischaemia with or without prior preconditioning was not associated positively or negatively with the final levels of injury (138). *These data suggest that MAPK activation is not related to myocardial protection or injury.*

3.2.5 Tyrosine kinases

It is generally accepted that activation of a member(s) of the tyrosine kinase protein family is required for preconditioning, because pre-treatment with general tyrosine kinase inhibitors such as genestein and lavendustin A block protection (139, 140). It is unknown however whether this tyrosine kinase acts in series (141) or parallel (142) with PKC.

3.3 End-effectors

3.3.1 The K_{ATP} channel

In 1983, Noma discovered the ATP-dependent potassium channel (K_{ATP}) using the patch clamp technique in isolated guinea pig ventricular myocytes (143). These channels were named "ATP-dependent" because of their inhibition in response to physiological concentrations of ATP. The name of this channel was subsequently changed to ATP-sensitive potassium channels after the discovery of a variety of endogenous modulators, including ADP, pH, fatty acids and various GPCR ligands (144).

3.3.1.1 Structure

K_{ATP} is a complex of two different proteins. One subunit is an inward rectifying potassium channel (Kir), which is thought to make up the pore as a complex of four Kir subunits (145). The sulfonylurea receptor (SUR) is believed to provide regulation by altering the sensitivity and response of the pore to pharmacological agents and ATP (145). Two subtypes of each subunit are currently known (Kir6.1, Kir6.2, SUR1 and SUR2), and it has been suggested that different combinations of these subunits exist in different tissue types, which are responsible for the divergence in channel properties between cell types (144).

3.3.1.2 Function

As well as the myocardium, K_{ATP} channels are found in other tissues, such as the pancreas, central nervous system, kidney and smooth muscle, where they have been implicated in insulin secretion, neurotransmitter release, renin secretion and vascular muscle relaxation, respectively (146). It is their effect in cardiac myocytes however, namely the rapid channel opening during ischaemia in response to falling ATP concentrations, that stimulated the interest of researchers investigating the pathogenesis of myocardial ischaemia.

3.3.1.3 Regulation

Inhibition of K_{ATP} is produced not only by ATP, but also by its non-hydrolysable analogues, suggesting phosphorylation is not critical (145). ADP also regulates K_{ATP} activity, possibly by competing for the ATP binding site, although another ADP site is thought to mediate weak agonist activity (145). Although phosphorylation is not required for inhibition, it is thought that ATP can prime K_{ATP} for opening by causing channel phosphorylation (147). This is because in the absence of ATP, channel "run-down" occurs, which can be re-activated by MgATP but not non-hydrolysable ATP analogues. At present however, it is unclear whether phosphorylation and dephosphorylation are involved in the mechanism of "run-down" and reactivation by MgATP. Many GPCR agonists, such as adenosine and acetylcholine, are known to increase the probability of K_{ATP} opening. It is thought that, through the activation of PKC, these agonists reduce channel sensitivity to ATP at the SUR (145).

3.3.1.4 The effect of K_{ATP} channel opening during myocardial ischaemia

The role of the K_{ATP} channel during ischaemia was tested using selective pharmacological blockers and openers of the channel in various models. The hypothesis at the time being that K_{ATP} channel openers would be protective by shortening action potential duration and therefore reducing calcium entry. This would protect by sparing energy utilisation by inhibiting contractile function in a manner

similar to L-type calcium channel blockers. The vasodilatory effect of K_{ATP} agonists forced the hypothesis to be tested in isolated heart preparations, where their direct cardioprotective activity could be ascertained. This hypothesis appears partially correct since numerous investigations were able to shown a cardioprotective effect of K_{ATP} openers in several species, including dogs, rats and rabbits (148-152). Moreover, protection is also seen when K_{ATP} openers are applied to isolated cells *in vitro* (153, 154), suggesting that their cardioprotective effect are, at least to an extent, imparted onto myocytes. The energy-sparing hypothesis for the mechanism through which protection is achieved, was however, later to be disproved (see section 3.3.1.6).

3.3.1.5 K_{ATP} and ischaemic preconditioning

The ability of K_{ATP} agonist to "mimic" preconditioning and the capacity of preconditioning triggers to activate K_{ATP} channels imply they may be involved in mediating protection in response to sublethal ischaemia. It was Gross's group that first demonstrated that K_{ATP} blockers abolish preconditioning using glibenclamide in a canine model (155). Various K_{ATP} antagonists have since been shown to abolish protection in a wide variety of models (144). In humans, preconditioning displays a similar dependence on K_{ATP} opening, as glibenclamide blocks the protection against ST deviation seen following coronary balloon occlusion (156). The opening of K_{ATP} channels following classical preconditioning is likely to be downstream from the adenosine receptor, since adenosine-mediated protection can be abolished with K_{ATP} blockers (157-159).

3.3.1.6 The mechanism of cardioprotection

Most K_{ATP} openers are potent vasodilators, therefore it is possible that they exert cardioprotective effects through coronary dilatation. This is unlikely however, because K_{ATP} opening is still protective when flow is kept constant (150), and isolated myocytes can be protected *in vitro* (153, 154). The decrease in action potential duration (APD) seen following K_{ATP} opening may preserve intracellular ATP by slowing myocardial contraction and thus ATP utilisation during ischaemia. Moreover,

K_{ATP} openers increase the time to ischaemic contracture and have been shown to directly preserve myocardial ATP content during ischaemia (160). This preservation however, appears unrelated to contractile function, since no negative inotropic effects are observed after K_{ATP} opening. Furthermore, the K_{ATP} openers cromakalim and BMS-180448 were found to have additional protective effects over cardioplegia, confirming that they function by a mechanism distinct from cardioplegia (161). This alternative mechanism is also separate for APD shortening, because doses of bimakalim could be used to reduce infarct size in dogs, which have no effect on epithelial monophasic APD (162). This dissociation of APD and protection implied that sarcolemmal K^+ currents where superfluous to the cardioprotective effect of K_{ATP} openers.

3.3.1.7 Mitochondrial vs. Sarcolemmal K_{ATP}

Other data, as well as the dissociation of APD and cardioprotection, exist to suggest sarcolemmal K_{ATP} activation does not contribute to protection. 5-hydroxydecanoate (5-HD), a K_{ATP} antagonist, has little effect on sarcolemmal channels but blocks the cardioprotective effects of a range of K_{ATP} openers (163, 164). Furthermore, pharmacological agents were developed that retain the glyburide-reversible cardioprotective activity of cromakalim while being relatively devoid of vasodilator activity (165). Interestingly these agents were poor at opening sarcolemmal K_{ATP} channels, as measured by single channel patches or whole myocyte K^+ currents (166). Taken together, the data described above shows a poor correlation between sarcolemmal K^+ current and cardioprotection in response to K_{ATP} channel openers or preconditioning.

In 1991, Inoue *et al.* discovered a potassium channel in mitochondria from patch clamp studies on fused mitoplasts (167). This channel was regulated by many ligands that regulate plasma membrane K_{ATP} channels and, as such, was termed the mitochondrial K_{ATP} channel (mitoK_{ATP}). Some ligands do however display specificity for mitochondrial over sarcolemmal channels. Diazoxide is about 1000 times more potent in opening mitoK_{ATP} than opening sarcolemmal K_{ATP} channels (144, 168).

Furthermore, Garlid's group (144) and others (164) reported that 5-HD, under appropriate conditions, always inhibits mitoK_{ATP} current but never sarcolemmal K_{ATP}. This differential selectivity provided an ideal opportunity to study the relative contribution of these different channels to ischaemic preconditioning and myocardial protection. Using these agents, Garlid's group showed that diazoxide was able to protect the isolated rat heart against ischaemia when used in the low micromolar range, at concentrations that are insufficient to activate sarcolemmal K_{ATP} current or shorten APD (163). Moreover, both glyburide and 5-HD completely abolish these protective effects of diazoxide (163) and protection in response to ischaemic preconditioning (169). These data have recently been confirmed by Marban's group using the structurally distinct agents HMR1098 and P-1075, which selectively inhibit and activated sarcolemmal K_{ATP} currents respectively. They found that, unlike 5-HD and diazoxide, these agents are unable to abolish or mimic the protection afforded by preconditioning (170). These data appear to exclude a role for sarcolemmal K_{ATP} channels in ischaemic protection and suggest that mitoK_{ATP} channels are the likely effectors of cardioprotection.

3.3.1.8 A trigger or effector?

The mitoK_{ATP} was, until recently, thought to be an effector of protection by either mitochondrial calcium homeostasis (171), preservation of the inter-membrane space (172), interaction with the cytoskeleton (173), or prevention of apoptosis (174). Recently however, studies from Downey's group have suggested that the K_{ATP} channel may serve as a trigger, rather than effector, of protection (175). This hypothesis was formed after the K_{ATP} channel blockers 5-HD and glibenclamide abolished preconditioning when given during the brief ischaemic stimulus and not during the lethal index ischaemia (176). This was supported by earlier work with diazoxide, a mitoK_{ATP} channel agonist, which protected when given prior to, but not during, lethal ischaemia (173). The sudden release of free radicals, as a result of potassium influx into mitochondria and subsequent uncoupling of the electron transport chain, has been proposed as a possible mechanism by which channel opening triggers protection.

3.3.2 A role for anti-apoptotic proteins?

Programmed cell death, or apoptosis, is believed to contribute to myocardial injury during ischaemia/reperfusion in animal models. Furthermore, apoptosis has been detected in combination with necrosis in the human heart following myocardial infarction (177). In isolated myocytes (178) and the whole heart (179, 180), preconditioning prior to ischaemia inhibits apoptosis following ischaemia/reperfusion. Apoptosis is initiated by a complex series of signalling events, which may be triggered during ischaemia and/or reperfusion. These signalling processes can be prevented or inhibited by other kinases that promote survival by antagonising the cell death signals. Preconditioning has been shown to upregulate the "anti-death" gene Bcl-2 (181) and downregulate the pro-apoptotic gene Bax (182). It is of course hard to dissociate cause from effect, as the severity of ischaemia may determine the extent of apoptosis following ischaemia, and therefore the decrease in apoptosis following preconditioning may simply be a consequence, rather than a cause, of protection. Nevertheless, the possibility that preconditioning initiates/attenuates signalling pathways that prevent/cause apoptosis is appealing from a clinical perspective, since it may be possible to mimic these effects pharmacologically following myocardial infarction.

3.3.3 Cytoskeletal strengthening

The cytoskeleton is a collection of polymeric structures consisting of microtubules, microfilaments and intermediate filaments. Disruption of cytoskeletal integrity during ischaemia may produce structural alterations in the cell that may contribute to cell injury and death (183). Members of the small heat shock protein family (namely HSP25/27 and αB-crystallin) are molecular chaperones that have been implicated in ischaemic preconditioning (184, 185). It is possible that oligomerisation of the small molecular weight (Mw) HSPs improves the mechanical stability of the cytoskeleton during preconditioning, thereby protecting myocytes during subsequent ischaemia (186, 187).

The exact mechanism by which preconditioning causes HSPs to protect is unknown, although p38-MAPK activation and the subsequent phosphorylation of MAPKAPK-2 is thought to be involved (128). Although these kinases stimulate HSP27 phosphorylation, it may be their effect on HSP27 translocation that promotes protection. In response to preconditioning in isolated cells (188) and the whole heart (189), HSP27 is translocated to the sarcomere. Furthermore, this relocalisation of HSP27 is blocked by p38 inhibition with SB203580, which is also known to abolish protection in similar models (127). It may be that translocation is dependent on phosphorylation, since p38 activation is known to phosphorylate HSP27. However, whilst some groups demonstrate phosphorylation and translocation of small heat shock proteins following preconditioning (185), others are unable to show either (190). Furthermore, whilst HSP27 overexpression is protective in adult cardiomyocytes (191), expression of non-phosphorylatable HSP27 mutants do not reverse this protection (192). Interestingly, HSP27 phosphorylation appears to prevent its oligomerisation (192, 193). Therefore, either oligomerisation of HSPs are not required for cytoskeletal strengthening or phosphorylation of HSPs/cytoskeletal strengthening is not required for protection. In summary, although preconditioning may phosphorylate/translocate small Mw HSPs, more direct evidence is required to link these processes to protection in whole heart models of preconditioning. These studies would obviously be facilitated by the generation of HSP27 knockout mice.

3.3.4 Ionic balance

3.3.4.1 Sodium-hydrogen exchanger

The hypothesis that activation of the sarcolemmal Na^+/H^+ exchanger may contribute to ischaemic injury was formed in 1985, one year prior to the discovery of ischaemic preconditioning, (194). Inhibition of exchanger activity with a range of highly selective antagonists such as HOE-694, HOE-642, and EMD-85131, have all been shown to afford a cardioprotective benefit in a variety of animal models of ischaemia and reperfusion (195). The question of whether ischaemic preconditioning modulates

Na^+/H^+ exchanger activity is more ambiguous. It is logical to assume that the Na^+/H^+ exchanger will be activated following preconditioning because intracellular acidosis, which occurs during the brief ischaemia, is able to trigger Na^+/H^+ exchanger activity (196). Furthermore, stimuli that mimic preconditioning, such as α_1-adrenergic receptor activation, also activate the Na^+/H^+ exchanger (196). It maybe that activation of the exchanger contributes to the reduction in intracellular acidosis by H^+ extrusion, although studies investigating this hypothesis have yielded contradictory data (197, 198). Regardless of the activation status of the exchanger following preconditioning, the critical question is whether Na^+/H^+ exchanger activity is required for protection. Although not all studies are in agreement, the large body of evidence suggests that inhibition of the exchanger does not prevent preconditioning (196). Actually, in the rat, concomitant inhibition of exchanger activity provides additive benefit against infarct size reduction (199) and enhanced contractile recovery (200). This suggests that, at least in the rat, the mechanisms of protection following ischaemic preconditioning and Na^+/H^+ exchange inhibition are distinct.

3.3.4.2 Calcium regulation

Cytoplasmic calcium overload during ischaemia or early reperfusion is though to contribute to myocardial cell injury. The sarcoplasmic reticulum (SR) is a principle regulator of cytosolic calcium. There have been several reports in the literature suggesting a role for the SR in the mechanism of preconditioning. In 1991, Tani *et al.* were the first to demonstrate a preservation of SR function after preconditioning, in that it lost less calcium during ischaemia and recovered more calcium following ischaemia (201). Two years later, Murphy's group showed a decrease in calcium concentration during ischaemia in preconditioning hearts, although whether this was a result of regulation via the SR or calcium exchangers is unknown (202). Although others have suggested a critical role for the SR as an effector of preconditioning (203, 204), a recent study appears to document preconditioning in the absence of a functional SR (205). Ryanodine, which locks the SR calcium release channel in the open state, inhibited the ability of SR to accumulate calcium but did not abolish the protective effect of ischaemic preconditioning (205).

Calcium is regulated by a number of mechanisms beyond the role of SR however, some of which have also been implicated in preconditioning. L-type calcium channels regulate sarcolemmal calcium influx into myocardial cells, thus the inhibition of these channels represents a viable target for protection. Early studies, assessing the ability of preconditioning to modulate the L-type calcium channel density during ischaemia, were unable to detect a difference between preconditioned and control hearts (206, 207). Interestingly, atrial muscle from patients taking L-type calcium channel antagonists could not be preconditioned by transient ischaemia, whereas control atrial muscle still exhibited preconditioning (208). The author's conclusion that, "this lack of preconditioning may be the basis of the increase cardiovascular mortality traditionally associated with calcium channel blockers in patients with coronary artery disease", has however been a topic of controversy (209, 210).

3.3.5 ATP preservation

When Murry and colleagues documented a delay in myocardial necrosis during ischaemia following preconditioning, they hypothesised that protection may result from a reduction in ATP depletion. The same group later showed that preconditioning had no effect on ATP levels at the end of ischaemia, although the utilization of ATP during ischaemia was attenuated (211). The exact effect on cellular ATP remains unclear since later reports showed end-ischaemic ATP levels are enhanced (212, 213), and ATP utilisation is accentuated (214), during ischaemia following preconditioning. Nevertheless the hypothesis that preconditioning "reduces myocardial energy demand during ischaemia, which results in a reduced rate of high energy phosphate utilization and a reduced rate of anaerobic glycolysis", proposed by Murry and colleagues (211) has received attention from other groups. If Murry's hypothesis were correct, it would be important to understand the mechanism whereby preconditioning reduces myocardial energy demand during ischaemia. As discussed above, although the initial hypothesis was that opening of sarcolemmal K_{ATP} channels would preserve intracellular ATP by shortening APD and inhibiting myocyte contraction, this theory has subsequently been disproved (section 3.3.1.6). Other theories have been proposed

for the reduction in ATP utilisation, which have subsequently been disproved. For example the concomitant myocardial stunning, that has been demonstrated following preconditioning, has been proposed to decrease contractile effort, and therefore decrease energy expenditure during the initial phase of lethal ischaemia (215). This theory now appears unlikely, because the protection against infarction following preconditioning disappears although myocardial stunning persists (216), and protection can be achieved in the absence of myocardial stunning (217). One of the most intriguing hypotheses however, concerns the mitoK_{ATP} channel and its ability to preserve the architecture of the inter-membrane space (IMS). This was proposed by Garlid who suggests that ischaemic preconditioning protects mitochondrial function *in situ* by increasing the V_{max} of respiration, the preservation of functional coupling between mitochondrial creatine kinase and adenine nucleotide translocase, and the absence of stimulation of respiration by cytochrome c. The net effects of these processes are preservation of cellular ATP levels during ischaemia and better contractile recovery upon reperfusion (172).

3.4 Summary of signalling during preconditioning

In summary, the proximal pathways that trigger preconditioning, such as G-protein-coupled receptor activation and protein kinase C translocation, are fairly well characterised. In contrast, the exact role of the distal effectors of preconditioning, such as mitogen-activated protein kinases and K_{ATP} channels, is more controversial (175, 218). An unbiased schematic of the signalling pathways implicated in preconditioning is depicted in Figure 1-8.

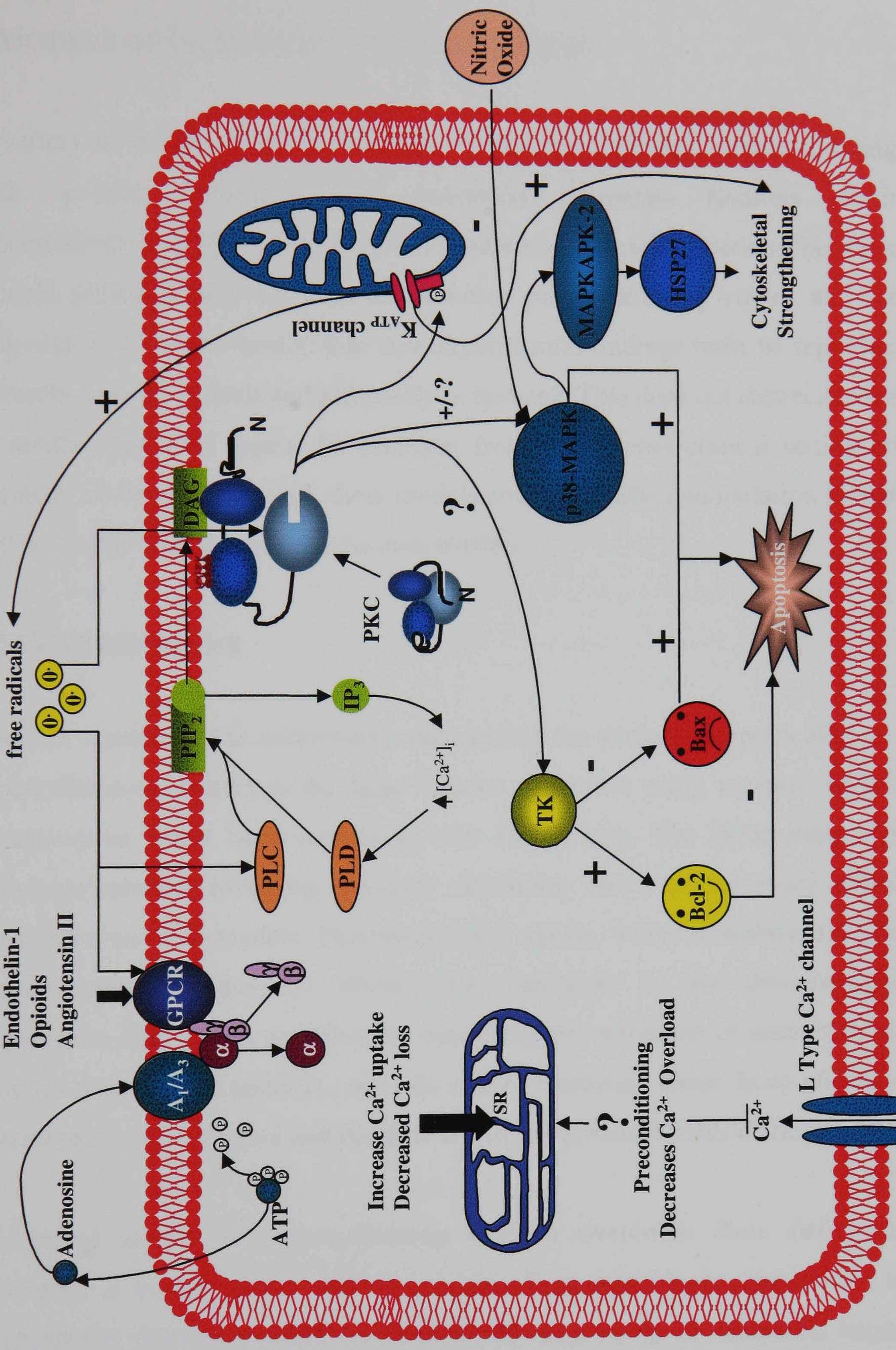

Figure 1-8: Signalling pathways implicated in early ischaemic preconditioning.

4 MODELS OF ISCHAEMIC PRECONDITIONING

A variety of models have been used to investigate ischaemic preconditioning, each with specific advantages and drawbacks. Disparate findings within the preconditioning field are frequently attributed to species variations between these models, although other concerns are maturity, preconditioning trigger, and choice of endpoint. It is for this reason that new experimental findings must be reproducible in different animals models and ultimately in humans. This does not depreciate the value of models that may appear far removed from the *in vivo* clinical setting however because, as will be discussed, these models enable specific manipulation of signalling pathways that would otherwise be inaccessible.

4.1 Cell-based models

There is currently little controversy surrounding the surface receptors able to trigger preconditioning, thanks to the large volume of studies using receptor agonists and antagonist in whole heart models *in vitro* and *in vivo*. The intracellular signalling pathways evoked following receptor activation have proved more difficult to investigate in these models. Pharmacological agents, where available, are often less specific and have systemic affects that complicate *in vivo* data interpretation. Associative data can be described by examining the activation of kinases in response to a preconditioning stimulus, but this cannot dissociate cause from effect, and the heterogeneity in cell types and depth of ischaemia present further difficulties.

Cell-based models of preconditioning help to overcome these deficiencies by providing a homogeneous cell type where it is possible to manipulate specific intracellular signalling pathways through the expression of dominant negative or constitutively active signalling proteins. For these and other reasons, various cell models have been developed to investigate preconditioning.

4.1.1 Immature cardiocytes

Mature (adult) cardiocytes die or dedifferentiate in culture and are relatively resistant to standard transfection techniques. This has caused some investigators to use immature cardiocytes models such as neonatal rat myocytes, embryonic chick myocytes and paediatric human cardiocytes (219). A study in chick embryonic myocytes showed that A_1 and A_3 receptor overexpression conferred endogenous protection and enhanced preconditioning-induced protection against simulated ischaemia, confirming the importance of these particular receptor subtypes (220). Neonatal rat cardiocytes have been used to investigate the contribution of PKC isoforms to simulated preconditioning. These studies have showed that specific PKCε inhibition abolished protection in response to preconditioning (107), whereas expression of an active PKCδ mutant provided protection against simulated ischaemia alone (221).

4.1.2 Mature cardiocytes

Adult cardiomyocytes have a rate of attrition as great as 30% following isolation, therefore models usually rely on the suspension of freshly isolated cells. The most commonly used is the model developed by Armstrong *et al.*, where lethal ischaemia is achieved by overlaying pelleted cardiomyocytes with mineral oil (59). Short periods of cell pelleting initiates preconditioning that, like preconditioning in the intact heart, is dependent on adenosine, PKC and K_{ATP} channels (219). In particular, these models have revealed protection against lethal simulated ischaemia in response to the activation of A_3 receptors (222), PKCε and ERK1/2-MAPK (223).

Cell cultures also enable voltage-clamp recordings in single cell electrophysiology. This approach has provided a link between the sarcolemmal K_{ATP} channel and PKC activation. In voltage-clamp studies in adult rabbit and human cardiomyocytes, activation of PKC, in the presence of low intracellular ATP, increased current through sarcolemmal K_{ATP} channels (224). Similarly, in rabbit cardiomyocytes there was an accelerated onset of current through the sarcolemmal K_{ATP} channel in response to

metabolic inhibition in the presence of both adenosine and PKC pre-activation, but not with either alone (225). This effect could be blocked in cell-lines by transfection of a dominant negative potassium channel subunit (226). Furthermore, a similar relationship is found between PKC activation and the inferred opening of a mitochondrial K_{ATP} channel from measurements of mitochondrial redox state (227).

These studies highlight the advantages of isolated cell models. These advantages are however at the expense of a cell phenotype that differs from the intact heart, and cannot be subjected to true ischaemia/reperfusion. For these reasons it is imperative that preliminary data obtained in these studies is later confirmed in more physiologically relevant whole heart models.

4.2 Animal models

4.2.1 *Whole heart preparations in-vitro*

The isolated whole heart has proved invaluable as a model of ischaemia/reperfusion injury since its first description by Langendorff in 1895 (228). The heart can be maintained in this mode for many hours by perfusion with blood from a donor animal or crystalloid buffer (Kreb's Henseleit Buffer). Ischaemia is mimicked in this model by restricting the flow of blood or buffer, through the whole heart (*global ischaemia*) or a coronary artery (*regional ischaemia*). Myocardial injury can be measured by infarct size determination with triphenyl tetrazolium chloride (TTC) staining of viable tissue, enzyme release into the perfusate, and recovery of left ventricular developed pressure (for full description see: Chapter 2, section 4.2). Preconditioning is best characterised in the isolated rat (60, 100, 229) and rabbit heart (54, 93, 230), although it has also been demonstrated in the guinea pig (231) and mouse heart (29, 232) *in vitro*. The assessment of protein modification/activation by preconditioning or ischaemia/reperfusion is simplified in perfused heart models because the risk zone after global ischaemia is simply defined as the whole heart. Furthermore, peripheral effects on the vasculature and other organs do not complicate pharmacological

approaches, it is relatively easy to implement, and less labour intensive than *in vivo* whole heart models.

4.2.2 Animal models in-vivo

The most clinically relevant model, outside of myocardial ischaemia in humans, is regional ischaemia in the anaesthetised whole animal. These open-chested models have been developed in the pig (142), dog (14), rabbit (66), rat (233) and mouse (234) to investigate ischaemic preconditioning *in vivo*.

4.3 Human models

Isolated cells provide an excellent, readily accessible means of examining preconditioning in humans. So far human ventricular cardiomyocytes (235), right atrial trabeculae (236), and paediatric myocytes (237) have all been shown to precondition successfully in culture. Moreover, they appear to be dependent on the same critical signalling pathways as animal models, such as adenosine and PKC (237).

A number of clinical settings enable the preconditioning phenomenon to be investigated in humans *in vivo*. These models, which include cardiac surgery and percutaneous transluminal coronary angioplasty (PTCA), will be discussed in more detail in the following sections.

5 ISCHAEMIC PRECONDITIONING IN MAN

Throughout the 1970s and 80s it was a goal of many academic cardiologists to discover a method of limiting infarct size following an ischaemic insult (10). It was in this light that Murry and colleagues published their seminal paper on ischaemic preconditioning (14). This remains today the most effective method of limiting infarction other than reperfusion. Whilst the intense research investment has led to a clearer understanding of the underlying intracellular pathways in animals, we must

now push to reinforce these theories in humans if the protection seen following preconditioning is to be translated into a clinical therapy.

5.1 Evidence for preconditioning in humans

The preconditioning phenomena has been observed in all species tested thus far including rabbit, rat, dog, pig and mouse. The most critical question, and perhaps the most difficult to answer due to ethical limitations, is "does preconditioning occur in humans?" At least five preconditioning models have been used to address this question.

5.1.1 Preinfarction angina

The situation where angina precedes ischaemia provides the ideal setting for a retrospective study into the beneficial effects of antecedent sublethal ischaemia. Most studies of this genre have demonstrated a beneficial effect of angina prior to myocardial infarction (238, 239). Benefit is defined in some studies as a reduction in enzyme-derived infarct size (239) with improved short term and long term prognosis (240), although this has been proposed to result from a greater chance of patency being achieved rapidly in the infarct related artery if there is antecedent angina. Protection may therefore result from facilitated thrombolysis of thrombosed atherosclerotic plaques in patients with unstable angina, instead of preconditioning of distal myocardium. Although Hata *et al.* have shown that ischaemic preconditioning improves vessel patency in a canine model (241), multivariate analysis of human studies suggest that, despite the improved vessel patency, angina itself is independently associated with improved survival at 5 years (240).

The best prospective data comes in an ancillary study to TIMI-9B, where 3002 patients were asked to report symptoms of angina before their infarct. This study showed that patients who reported symptoms within 24 hours of infarction had a lower 30-day event rate than those with angina greater than 24 hours before the event (242). This suggests a role for either classical and/or delayed preconditioning. The

main problem in all these studies is that patients with more frequent angina differ in respect to concomitant medication, collateral circulation and time to presentation after onset of chest pain. All these factors have been shown to alter prognosis, which only serves to confound data interpretation.

5.1.2 Percutaneous transarterial coronary angioplasty

Percutaneous transarterial coronary angioplasty (PTCA) provides a controlled model of ischaemic preconditioning where the first balloon inflation mimics the preconditioning stimulus and lessens ischaemic parameters such as lactate production, ST deviation, and chest pain during subsequent inflation. The primary concerns with this model are the duration of balloon inflation and the presence of a collateral circulation. In studies that report no benefit the first inflation lasts around 2 minutes (243), whereas a stimulus of at least 3 minutes is required to protect in animal models (244). When protection is seen it is not clear whether this is a result of collateral recruitment following the first inflation. However, a recent study from our laboratory has shown protection during PTCA in the absence of any significant collateral recruitment, providing the most robust evidence of ischaemic preconditioning in man (245).

Pharmacological manipulation of the preconditioning pathway has also been used to trigger protection against the first inflation during PTCA. Using a 5-minute intracoronary infusion of adenosine, Lesser *et al.* have shown protection is even greater than with ischaemia (246). Moreover, infusion of an adenosine antagonist (247), or pretreatment with a K_{ATP} channel blocker (156), is able to prevent the preconditioning effect of the first balloon inflation.

5.1.3 Warm-up angina

Warm-up or walk-through angina describes the ability of patients to exert for longer during a second exercise than the first, when the two are separated by no longer than

60 minutes rest. The mechanism of the induced tolerance has been ascribed to ischaemic preconditioning, although this topic has received much debate (248).

Traditionally, warm-up angina was attributed to coronary vasodilatation, with perhaps the concomitant opening of collateral vessels to support the ischaemic myocardium (249). This hypothesis was accepted even though arterial vasodilators such as aminophylline had little effect on exercise tolerance (250), and the presence of collaterals on angiography where not predictors of warm-up angina (251). A study from our laboratory on patients that show no collateral recruitment during PTCA, demonstrates that these patients still exhibit the warm-up phenomenon on exercise (245). Moreover, the time frame of warm-up is congruous with preconditioning since the second exercise reproducibly exceeds the first provided that the two are separated by a rest period of no longer than 60 minutes.

5.1.4 Cardiac surgery

Cross clamping of the aorta during coronary artery bypass isolates the coronary circulation to allow fashioning of the grafts. This model has been exploited by Yellon *et al.* who were able to show that two 3 minute cross clamping periods slows the rate of ATP depletion and attenuates troponin T release during a 10 minute ischaemic stimulus (252). Some groups do not find an additive protective effect of preconditioning in this model (253). Since cardioplegic solutions are known to preserve ATP by reducing cellular metabolic demand, it is possible that they are acting in the same fashion as ischaemic preconditioning, although Lee *et al.* did successfully precondition using a presurgical infusion of adenosine (254).

5.1.5 Isolated muscle and cultured myocyte experiments

Ischaemic preconditioning has been demonstrated *in vitro* using small atrial trabeculae harvested during cardiac surgery (154). This protection, measured as an improvement in contractile function, is also dependent on the activation of PKC and the opening of K_{ATP} channels (154). Moreover, protection occurs in response to

preconditioning of human adult cardiac myocytes, which have been successfully cultured from patients with congenital heart disease (235).

5.2 Therapeutic potential of ischaemic preconditioning

5.2.1 Acute myocardial infarction

The most obvious therapeutic use for a cardioprotective treatment against ischaemia is in the treatment of acute myocardial infarction. Unfortunately the need to predict the onset of ischaemia poses a difficult challenge. It is possible that preconditioning initiates a signalling pathway that protects the heart during ischaemia and/or reperfusion. In this instance, pathways could be activated or inhibited after the onset of ischaemia. It has been suggested that adenosine is required as a trigger before ischaemia and a mediator during ischaemia. Todd *et al.* showed in a rabbit model that an adenosine agonist given *during* the index ischaemia exerted a cardioprotective effect and reduced infarct size (255). However, although unrelated groups show a similar benefit (256, 257), others in similar models show no benefit of adenosine infusion (258, 259). This technique has been tested in man in the AMISTAD trial, which was a prospective, open label trial of thrombolysis with randomisation to adenosine or placebo in 236 patients within 6 hours after the onset of infarction (260). In the study adenosine caused a 33% reduction in infarct size, which supports the need for a large-scale clinical outcome trial.

It has been suggested in animal models that protection is achieved, at least in part, during reperfusion following index ischaemia. For example, in canine hearts blockade of the adenosine receptor during reperfusion reverses the infarct-limiting effect of preconditioning (261). Preconditioning has also been proposed to attenuate oxidant stress during reperfusion (262). It may therefore be possible to harness the potential of ischaemic preconditioning against myocardial infarction without the need to predict the onset of ischaemia.

5.2.2 Prophylactic myocardial protection

If the heart was in a permanently protected state this would negate the need to predict the onset of ischaemia. The feasibility of "bottling protection" so that a drug could be administered to an "at risk" population, thus placing their heart in a permanently protected state, has been discussed (263). Unfortunately adenosine, one of the obvious candidates, if given as repeated infusions, downregulates its receptor (264). It may be possible to overcome this problem by including an adenosine free period in the regime (265).

If, as discussed previously, adenosine is protective during ischaemia and/or reperfusion it may be possible to enhance endogenous adenosine during these periods. Dralfalazine inhibits nucleoside transport, thus bolstering adenosine concentrations during low flow ischaemia and, in isolated porcine hearts, elicits a cardioprotective effect (256).

The same principle can be applied to other triggers of preconditioning. Angiotensin converting enzyme (ACE) inhibitors augment bradykinin levels through inhibition of their breakdown. Large clinical trials such as GISSI-3 (266), ISIS-4 (267), SAVE (268), and SOLVD (269), which were designed to assess the ability of these agents as effective heart failure drugs, revealed unexpected findings. Patients who received these drugs post-infarction had a decreased incidence of adverse ischaemic events and a significant reduction in early mortality, at a time when the remodelling effects had probably not yet become effective.

ACE inhibitors, given prior to ischaemia in animal models, lead to a reduction in infarct size, decrease in reperfusion-induced dysrhythmias and improved contractile function compared to controls (270-272). It is likely that in future patients with coronary artery disease receive ACE inhibitors and not just those post myocardial infarction.

Pharmacological manipulation distal to the receptor may also represent a viable clinical target. Nicorandil, a drug used to treat angina, has two moieties, a nitrate group and a K_{ATP} opener. Although it's use in angina is primarily due to the vasodilatory effect of the nitrate group, it is its effect on K_{ATP} channels that has generated most interest. A nicorandil infusion prior to ischaemia protects against stunning and infarction in a variety of animal models (273, 274). More interestingly, these effects are not mimicked by pure nitrate donors and are abolished by K_{ATP} channel antagonists (275, 276). In a recent placebo controlled study, nicorandil, administered to patients with unstable angina, reduced myocardial ischaemia and dysrhythmias (277). The authors suggested that pharmacological preconditioning was responsible for these effects.

5.2.3 Surgically induced ischaemia

The ischaemia deliberately induced during cardiac surgery offers the ideally controlled setting to precondition the myocardium. The ischaemia is anticipated, of short duration and always associated with reperfusion. Very successful use of buffered hypothermic solutions means that fortunately irreversible myocardial injury rarely occurs. In complex procedures however, troponin-T release has been detected, indicating discrete necrosis, which may be attenuated by prior preconditioning in combination with cardioplegia.

Transplant surgery may represent another therapeutic target for preconditioning. For the heart to remain suitable for transplantation it should be stored hypothermically for no longer than 4-6 hours. Animal studies suggest that preconditioning may increase this therapeutic window, which may decrease the current burden on donor hearts (273, 274).

6 AIMS AND SCOPE OF THE THESIS

Classical ischaemic preconditioning provides a powerful means of protecting the heart against ischaemia. A better understanding of the underlying signalling pathways may enable protection to be "bottled" pharmacologically. PKC is a well-characterised family of signalling proteins involved in ischaemic preconditioning, but the involvement of specific PKC isoforms is more controversial. Furthermore, the kinases downstream of PKC are also less well understood. A better understanding of the role of individual PKC isoforms and their downstream targets should reveal more specific therapeutic targets. The purpose of the studies described within this thesis is to further characterise the signalling proteins involved in mediating protection in response to ischaemic preconditioning.

The specific questions that the studies contained within this thesis were designed to answer were:

- What kinases are activated during preconditioning in neonatal cardiac myocytes?
- Do kinases activated during preconditioning contribute to protection?
- Do isolated mouse hearts precondition?
- Is PKCε necessary for preconditioning in the mouse?

Chapter 2. Materials and Methods

The constituents of all solutions stated within this methods chapter can found in the appendix section of this thesis (page 199).

1 VECTOR PRODUCTION

1.1 Virus production

1.1.1 Principles of adenoviral growth and purification

The recombinant adenovirus has essential regions deleted from its viral genome and as such can only be grown efficiently in cells expressing proteins that complement these deleted regions. The HEK 293 cell line was used, since these have been shown to allow growth of such viruses (278). After infection into the cell, the virus replicates leading eventually to cell death. Immediately prior to cell death (when previously attached cells begin to float in the media), the cells are harvested and lysed by repetitive freeze-thaw cycles. The virus can then be purified on caesium chloride gradients from not only cellular constituents, but also empty capsid and protein, since viable virus has a higher buoyant density. A final step of dialysis allows the removal of caesium chloride from the viral preparation.

1.1.2 Generation and growth

HEK 293 cells were cultured using standard tissue culture techniques. When cells were 80-90% confluent (typically in 10 × 140mm dishes), they were washed briefly in PBS followed by addition of 5mls of serum free media (SFM) containing recombinant adenovirus at an multiplicity of infection (MOI) of 10 ($\approx 1 \times 10^7$ plaque forming units (pfu) per dish). Cells were then returned to the incubator for 1 hour prior to addition of 10mls of full growth media (FGM). Cells were then cultured until a cytopathic effect was seen in the majority of cells, typically 2-3 days post infection.

1.1.3 Purification

Cells were harvested in virus storage media (10mM Tris [pH 7.4], 1mM $MgCl_2$ and 10% (v/v) glycerol) and subjected to 3 freeze-thaw cycles (liquid nitrogen to a 37°C water bath). The lysate was then spun at 800g to remove cellular debris (3000rpm, Universal 16, Heffion Ltd., Tattlingem, Germany). Supernatant was then transferred onto a 1.25g/ml (4mls) to 1.4g/ml (4mls) caesium chloride step gradient and centrifuged at 150,000g using a SW41Ti swing-out rotor (Beckman Ltd., Paolo Alto, California, USA) for 2 hours at 20°C. The upper viral band was then removed and further purified on a second caesium chloride gradient (1.35g/ml) at 150,000g using a 70Ti fixed angle rotor (Beckman Ltd.) for 18 hours at 20°C. The purified viable virus was dialysed using 2000 MWCO dialysis cassettes (Slide-A-Lyzer® 2K, Pierce, Rockford, Illinois, USA) with virus storage medium at 4°C over 24 hours with 3-4 dialysis changes. Purified virus was then stored, in aliquots of 1 and 10µl, at –70°C.

1.1.4 Screening to exclude wild type contamination

Recombinant adenoviral preparations were added to HeLa cells (in 100mm dishes) in 4mls of SFM, at an MOI of approximately 20. After 2 hours an additional 4mls of FGM was added. Three days later the media was aspirated and centrifuged to remove cellular debris.

HeLa cells, grown to 80-90% confluency in 24 well plates, were exposed to 300µl of supernatant. Six hours later, cells were washed briefly with PBS and 0.5mls of FGM was added. At day 4, cells were microscopically examined for a cytopathic effect. The presence of wild type contamination will be detected as a cytopathic effect, since wild type adenovirus has no deletions within the viral genome and thus does not require complementation.

1.1.5 Plaque titration

Following growth to confluence in a 1 × 75cm^2 tissue culture flask (Nunc, Rosklide, Denmark), HEK 293 cells were split into four 6-well plates. 24 hours later, 100µl of virus was serially diluted (1:10) into 1ml of SFM. Virus was pipetted into 6-well plates, from most dilute upwards, and left for 1 hour, at which point 1ml of FGM was added to each well. 48 hours later, viral titre was assessed by counting the number of plaques using a light microscope at 10× magnification (Nikon TMS, Nikon, Japan).

1.2 Expression plasmid production

1.2.1 Principles of plasmid DNA growth and purification

The plasmid DNA is introduced into *Escherichia coli* (DH5α) by first using divalent cations to attach the DNA to the bacterial membrane. The membrane is then permeabilised by rapid warming, which allows the DNA to be taken into the cell. The bacteria are then grown in media supplemented with an antibiotic, the resistance to which is encoded within the plasmid DNA, thus allowing selective growth of bacteria containing the plasmid DNA. Following bacterial growth and amplification, plasmid DNA is recovered following a series of steps that denature the plasmid and chromosomal DNA. Plasmid DNA can be purified from chromosomal DNA since only covalently closed plasmid DNA is rehybridised, whereas chromosomal DNA and cellular debris remain insoluble. Further purification rids the preparation of remaining protein and RNA.

1.2.2 Competent cells

Competent cells were prepared by growing a single colony of DH5α bacteria overnight in 10mls of LB media in an orbital incubator (Innova 4300, New Brunswick Scientific Co. Inc., Edison, New Jersey, USA) at 200rpm and 37°C. 100µl was transferred into 100mls of LB media and incubated for approximately 2 hours until swirls of bacteria were visible (absorbance at 600nm ≈0.4-0.6). The culture was then

centrifuged at 4500g (3000rpm, Universal 16, Heffion Ltd., Tattlingem, Germany) and the pellet was resuspended in 10mls of 100mM $CaCl_2$ at 4°C and left on ice for 1 hour. The bacteria were pelleted again and resuspended in 4mls of 100mM $CaCl_2$.

1.2.3 Transformation of competent cells

100µl of competent cells were incubated with 100ng of plasmid DNA (in 10µl) for an hour at 4°C prior to heat shock at 42°C for 90 seconds. The mixture was then placed on ice for a further 30 minutes, at which time they were plated on LB-agar plates, containing ampicillin at 100µg/ml, and incubated overnight at 37°C.

1.2.4 Small-scale preparation of plasmid DNA

A single colony was picked and incubated overnight in 10mls of LB, containing ampicillin at 100µg/ml, in a 50ml conical flask at 200rpm in an orbital incubator at 37°C. Bacteria were pelleted as before and resuspended in 200µl of TE pH 8.0 followed by addition of 400µg/ml of RNAse A. 200µl of solution 2 was then added, the suspension vortexed and kept at room temperature for 5 minutes. 200µl of solution 3 was then added and the suspension spun at 15,000g in a microfuge (13,000rpm, Heraeus Biofuge pico, Heraeos Instruments, Germany). The supernatant was transferred to a new hours tube and 1 volume of isopropranolol added and incubated on ice for 10 minutes prior to centrifugation again. The pellet was resuspended in 200µl of TE and phenol: chloroform cleaned.

1.2.5 Phenol: chloroform extraction of DNA

Phenol treatment causes protein denaturation, thus allowing protein removal. DNA is then purified from phenol by chloroform: isoamylalcohol washes, and finally chloroform is removed by ethanol precipitation. Phenol was added to the DNA in solution (1:1, v/v), the mixture vortexed and the upper aqueous layer removed, with care being taken not to remove denatured protein at the interface between the aqueous and phenol layer. The aqueous phase is then mixed with an equal volume of

chloroform: isoamylalcohol, vortexed and the aqueous layer removed again and ethanol precipitated.

1.2.6 Ethanol precipitation

2.5 volumes absolute ethanol and 0.1 volumes of 3M sodium acetate (pH 5.2) is added to the DNA solution and the mixture left on ice for 10 minutes. Following centrifugation at 13,000rpm in a microcentrifuge, the pellet was washed in 70% (v/v) ethanol to remove traces of salt. After further centrifugation, the DNA pellet was dried to remove all trace of ethanol and then resuspended in the appropriate volume of DI H_2O.

1.2.7 Large-scale preparation of DNA

Frozen stocks were used to inoculate 10mls of LB medium (with 10µl ampicillin at 100mg/ml), which was grown for 6 hours in an orbital incubator at 37°C. The 10mls culture was then added to a 2 litre conical flask containing 400mls of LB medium, with 100µg/ml of ampicillin, and placed in an orbital incubator overnight at 200rpm and 37°C. The culture was centrifuged at 5000rpm in a Sorvall GSA rotor (Sorvall Ltd., Stevenage, UK) for 10 minutes at 4°C and the pellet was resuspended in 20mls of solution 1. The cells were lysed and DNA denatured by addition of 40mls of solution 2. After leaving cells on ice for 10 minutes, 30mls of solution 3 was added and the cells incubated on ice for a further 10 minutes to aggregate proteins and promote intrastrand aggregation of chromosomal DNA, whilst plasmid DNA rehybridises.

Cellular debris was pelleted by centrifugation as before, the supernatant removed to another centrifuge pot and 50mls ice-cold isopropranolol added. Following incubation on ice for 10 minutes the suspension was centrifuged at 9000rpm in a Sorvall GSA rotor for 15 minutes at 4°C. The supernatant was discarded, leaving about 1ml into which the pellet was resuspended and transferred to a 1.5ml Eppendorf tube. Following a 10-minute centrifugation in a microfuge at 4°C, the pellet was

resuspended in 720µl of TE buffer, to which 1.26g of caesium chloride and 120µl of 20mg/ml ethidium bromide was added. Once dissolved, the tubes were left overnight in the dark at room temperature. The tubes were centrifuged at 13,000rpm in the microfuge for 30 minutes to remove residual protein, which forms as a precipitate. The DNA solution was underlayed below a 65% (w/v) caesium chloride gradient, and the gradient centrifuged at 100,000rpm in a TLA100.4 rotor using a Beckman Optima TI ultracentrifuge (Beckman Ltd., Paolo Alto, California, USA) for 4 hours, during which a gradient is formed in the caesium chloride, allowing the separation of DNA. The plasmid DNA (red band) was recovered and washed repeatedly in water-saturated butanol to remove residual ethidium bromide. The DNA-containing aqueous phase was phenol: chloroform cleaned, ethanol precipitated, and the resulting DNA quantified.

1.2.8 Quantification of DNA

Plasmid DNA was diluted 100 fold in a total volume of 500µl of DI H_2O and the absorbance at 260 and 280nm measured with a spectrophotometer (Jenway 6105 U.V./Vis Spectrophotometer, Jenway Ltd., Dunmow, Essex, UK), after initial standardization to a blank reading with DI H_2O alone. The DNA concentration (µg/µl) can be calculated by: 5(absorbance at 620nm [units]), since at 260nm an absorbance of 1 unit is equivalent to 50µg of DNA. The purity of DNA is calculated by 260nm: 280nm ratios, which were always between 1.8 and 1.9.

2 NEONATAL RAT VENTRICULAR MYOCYTE CULTURE

2.1 Isolation and purification

Neonates were rinsed briefly in 70% (v/v) ethanol and a midline incision made through the sternum. After removal of the heart, the ventricle was trisected into ADS buffer (see Appendix, section 1.2.1). Following removal of 20-30 hearts, the ADS was aspirated and replaced by 7mls digestion buffer (ADS + 0.5 mg/ml collagenase

and 0.6 mg/ml pancreatin). The digestion mixture was then incubated at 37°C for 7 minutes. Thereafter, the buffer was aspirated and replaced by another 7mls digestion solution and digested again at 37°C for a further 7 minutes. The digestion buffer is discarded following these first two digestions, however the processes was repeated a further 6 times with 15-minute digestion periods. After each digestion cycle the 7mls of buffer is added to 2mls of FCS, the mixture centrifuged at 1000rpm (Universal 16, Heffion Ltd., Tattlingem, Germany) for 6 minutes, the supernatant discarded and the pellet pooled into 4mls of FCS. The pooled cells were then centrifuged and resuspended in 50mls plating media. The cell suspension was pre-plated for 30 minutes in 90mm dishes (4 dishes with 12.5mls/dish) to remove fibroblast contamination. The media (containing <5% fibroblasts) was then retrieved and placed in 6-well gelatin-coated plates (2mls/well). These gelatin-coated plates were prepared from standard 6-well plates exposed to 1ml of 1% gelatin (v/v, in PBS) two hours before use (the gelatin solution is aspirated immediately prior to myocyte plating).

2.2 Culture

Myocytes were cultured in plating medium for 24 hours following isolation at 37°C in room air containing 5% CO_2. Media was then aspirated and, following a brief wash with PBS, cells placed in 2mls maintenance media. Cells were cultured in maintenance media at 37°C for at least 24 hours prior to use.

2.3 DNA transfection

2.3.1 Adenoviral infection

One day post-isolation, myocytes, grown in 6-well plates to 70-80% confluency, were incubated with 1ml of SFM containing adenovirus at 1×10^7 pfu (MOI ≈10) for 1 hour at 37°C in room air containing 5% CO_2. The cells were washed briefly in PBS and cultured in 2mls maintenance medium containing 1% (v/v) FCS for an additional 48 hours at 37°C in room air containing 5% CO_2.

2.3.2 Plasmid transfection

Cardiocytes grown in 6-well plates at 70-80% confluency were transfected with pCAGGS expression plasmid by, unless otherwise stated, an integrin-targeting peptide-mediated transfection procedure described previously (279). The peptide used was P6, which has a polylysine DNA binding region (16 residues) and an $\alpha_v\beta_1$ integrin-targeting head group (GACRRETAWACG). The peptide-Lipofectin complexes were prepared by mixing 40µl peptide (0.1%, w/v) and 0.75µl Lipofectin (Life Technologies Ltd., Paisley, UK). DNA (0.01%, w/v) in optimem was added to peptide-Lipofectin complex at a ratio of 2.5:1 (v/v). DNA-peptide-Lipofectin complexes were allowed to stand for 1 hour at room temperature before use. 100µl of this mix was diluted to 1ml in optimem and added to one well of a 6-well plate. Cells were then incubated overnight at 37°C in room air supplemented with 5% CO_2. Thereafter complex/optimem was removed and replaced with maintenance medium containing 1% (v/v) FCS, and the cells were returned to the incubator. Cell extracts were assayed for protein 48 hours post-transfection. By using pCAGGS-GFP as a reporter, transfection efficiency was consistently between 20 and 30%.

2.4 Principles of simulated ischaemia

In 1994, David Hearse in his capacity as editor of Cardiovascular Research invited 33 eminent cardiologists to write a simple definition of ischaemia (1). The response ranged from 3 words (supply-demand imbalance) to 404 words, proving that the term ischaemia was difficult to define without ambiguity. I would suggest that myocardial ischaemia is initiated by an inadequate blood supply to the heart. The consequence of the limited blood supply is that oxygen and nutrient availability is inadequate and tissue metabolites are insufficiently washed out. Thus, in the words of Dr Howard Morgan, "Ischaemia may be simulated in organ baths or tissue cultures by reducing oxygen and nutrient supply and adding metabolic products" (1). The model of simulated ischaemia used in the studies described within this thesis comprises hypoxia in a buffer lacking glucose but containing high potassium and lactate at low

pH. This therefore simulates oxygen and nutrient deprivation whilst mimicking the extracellular milieu of true ischaemia *in vivo*.

2.5 Ischaemia simulation

The cells were washed once with PBS before addition of 1ml of ischaemia buffer (IB: 118mM NaCl, 24mM $NaHCO_3$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, 0.5mM sodium·EDTA·$2H_2O$, 20mM sodium lactate, and 16mM KCl, pH 6.2), pre-gassed with 5% CO_2, 95% argon. On addition of ischaemia buffer spontaneous contraction within the monolayer ceased. Cells were then transferred to anaerobic GasPak pouches (Becton Dickinson, Maryland, USA) and incubated at 37°C for up to 6 hours. The O_2 content of the atmosphere inside the pouches was <1% for the duration of the experiment as measured by an anaerobic indicator.

2.6 Principles of cell viability assays

A number of endpoints can be used to assess cell death in cultured cells. We have chosen to use 3 separate endpoints that assess both membrane disruption and metabolic activity. One of the major forms of cell death during ischaemia and/or reperfusion is necrosis, during which the cell membrane integrity is lost and the cell constituents are leached to the extracellular milieu. Two enzymes, CK and LDH, can be detected in the buffer surrounding cells during ischaemia and quantified as a measure of membrane integrity or necrosis. Concomitant with necrosis, apoptosis has also been suggested to contribute to injury during ischaemia/reperfusion (177). Unlike necrosis, the cell membrane remains intact during apoptosis, however the mitochondrial membrane potential is similarly dissipated. The tetrazolium component of 3-(4,5-dimethylthiaziazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) is reduced in metabolically active cells to a blue formazan dye, hence the photometric quantification of tetrazolium reduction at the end of reperfusion provides an assay for live cells.

2.7 Cell viability assays

2.7.1 Measurement of enzyme release

On opening the anaerobic pouches (re-oxygenation) 200µl samples of the ischaemia buffer were gently collected for the determination of CK and LDH. The following day a spectrophotometric CK and LDH enzyme assay was performed with Boehringer Mannheim (MPR-1) and Sigma (TOX-7) assay kits respectively.

2.7.2 MTT bioreduction assay

After simulated ischaemia, cells were re-oxygenated in maintenance medium containing 1% (v/v) FCS. After 2 hours, medium was aspirated and cells incubated in 500µl PBS containing 2.5mg MTT for 30 minutes at 37°C in room air containing 5% CO_2. Thereafter the reaction was terminated by addition of 500µl solubilisation solution (0.1 mol/l HCl, 10% (v/v) triton X-100, in isopropranol) and the absorbance of the lysate was recorded at 570nm using a spectrophotometer, after initial standardization to a blank reading with DI H_2O alone.

3 PROTEIN DETECTION AND QUANTITATION BY WESTERN BLOTTING

3.1 Principles of SDS-Page and Western blotting

During SDS-page, proteins are separated according to molecular mass by electrophoresis through Polyacrylamide gels. The relative distance that a protein migrates is dependent on: (i) the pore size of the polyacrylamide gel, where pore size decreases with increasing acrylamide concentration, (ii) the effective radius of the protein, which is determined by its quaternary structure, and (iii) the native charge of the protein. It is common to denature proteins with a reducing agent in the presence of excess SDS, which acts to unfold the protein and cause it to bind SDS, thus conferring an essentially identical negative charge on all proteins. This has the effect of reducing the importance of quaternary structure and native charge, leading to a separation that

is mainly dependent upon molecular size. Following separation, proteins are transferred to nitrocellulose by applying an electric field perpendicular to the plane of the gel. The proteins become immobilized within the nitrocellulose matrix, whilst epitopes still remain accessible to immunoglobulins allowing immunodetection. The nitrocellulose membrane containing the immobilized protein is blocked, to prevent non-specific antibody binding, before exposure to primary antibodies specific for the protein of interest. The subsequent addition of horseradish peroxidase-conjugated immunoglobulins, raised against the primary antibody species, localises horseradish peroxidase to the protein of interest. Luminol is then added to the membrane in the presence of hydrogen peroxide. The peroxidase activity with the membrane catalyses the oxidation of luminol, which immediately after oxidation is in an excited state and emits light whilst decaying to the ground state. This light emission can be recorded on photographic film.

3.2 Preparation of protein samples

3.2.1 Cell cultures

Cells were washed twice in ice-cold PBS, before addition of 100µl of boiling two-times sample buffer (2×SB) per well of a 6-well plate. Cells were quickly removed into microcentrifuge tubes, 11µl of 2-mercaptoethanol added and the samples boiled for 5-10 minutes. 1-2µl of bromophenol blue dye was added and the samples centrifuged for 2 minutes at 13,000rpm in a microfuge (to remove insoluble material) prior to loading on SDS gels.

3.2.2 Isolated mouse hearts

Hearts were crushed under liquid nitrogen using a micro-pestle and motor. 30mg of heart sample was then weighed, placed in 350µl of boiling 2×SB containing 10% (v/v) 2-mercaptoethanol and boiled for 5-10 minutes. 1-2µl of bromophenol blue dye was then added and the samples centrifuged for 2 minutes at 13,000rpm in a microfuge prior to loading on SDS gels.

3.3 Preparation of SDS-Page gels

Polyacrylamide gels were poured between glass plates separated by 1mm spacers. The gels were formed of a lower "resolving" gel and an upper "stacking" gel into which wells are set to allow sample loading. The lower gel (pH 8.8) was prepared by mixing a 30% (v/v) acrylamide solution, TRIS-buffered SDS solution (to pH 8.8), and DI H_2O to give either a 10% or 12.5% acrylamide mixture. Prior to pouring, acrylamide polymerisation and cross-linking was induced by addition of ammonium persulphate (APS) and NNNN-tetraethylethalinediamine (TEMED). Water saturated butanol was overlaid whilst the gel polymerised to prevent drying.

Once the lower gel had polymerised, the butanol was washed off and the stacking gel poured. The upper stacking gel (pH 6.8) was prepared by mixing a 30% (v/v) acrylamide solution, TRIS-buffered SDS solution (to pH 6.8), and DI H_2O to give a 5% acrylamide gel mixture. The gel was polymerised by addition of APS and TEMED and poured on top of the resolving gel. Toothed plastic combs were inserted and removed after polymerisation to leave rectangular indentations to load samples. The exact constituents of the gel can be found in Table 7-1 (Appendix, page 208).

3.4 One-dimensional separation of proteins in SDS-page gels

Samples were loaded into their respective wells and a voltage of 120V applied using a Powerpac 300 (Biorad, Herts, UK). The negatively charged proteins migrate towards the lower reservoir (anode). Once the proteins had left the stacking gel (≈15 minutes) the voltage was increased to 150V until the dye front, the fastest migrating portion of samples, reached the bottom of the resolving gel. The resolving gel was then removed and used for either Western blotting or visualization of proteins by coomassie staining.

3.5 Coomassie staining of SDS-page gels

Coomassie brilliant blue (BDH, Dorset, England) binds to proteins and can quantitatively detect protein levels between 0.5 and 15µg (280). The resolving gel was gently agitated in coomassie staining solution for 1 hour, after which it was transferred to destain solution and left shaking at room temperature overnight, to remove the non-specific background staining.

3.6 Western blotting

Proteins were transferred to nitrocellulose membranes (Hybond C, Amersham, Bucks, UK) by overlaying nitrocellulose on the resolving gel, taking care to exclude air bubbles. The membrane and gel were then sandwiched between filter papers inside a plastic cassette within a blotting tank (BioRad, Herts, England) containing transfer buffer (0.025M Tris, 0.5M Glycine, and 20% (v/v) methanol). A current of 150mA was applied, with the anode placed on the nitrocellulose side, for 2 hours to allow the transfer of negatively charged proteins onto the nitrocellulose membrane. Following transfer, resolving gels were stained with coomassie blue to confirm efficient protein transfer.

Membranes were blocked against non-specific binding of antisera by incubation in blocking solution (5% (w/v) dried skimmed milk powder, 0.1% (v/v) Tween-20, in TBS) for 1 hour with gentle agitation at room temperature. Following a brief wash in TBS, containing 0.1% (v/v) Tween-20 and 0.1% (w/v) milk powder (TBST), membranes were incubated with the relevant primary antisera. For specific conditions of primary antibody incubations see the *specific methods* category of the relevant chapter (and Table 1-1; Appendix, page 212). Following antibody binding, membranes were washed in TBST for 10 minutes, four times. The membranes were then incubated with horseradish peroxidase conjugated secondary antibody. For specific conditions of secondary antibody incubations see the *specific methods* category of the relevant chapter. Following four further washes for 10 minutes in

TBST, membranes were developed using an enhanced chemiluminescence technique (Amersham, Bucks, England).

4 LANGENDORFF PERFUSION OF ISOLATED MOUSE HEARTS

4.1 Maintenance of the transgenic mouse colony

4.1.1 Breeding

Mice heterozygous for a disruption within the *pkc-ε* alleles were initially mated to produce either wild type (+/+), heterozygous (+/-) or knockout (-/-) offspring. Thereafter (+/+) × (+/+), (+/-) × (+/-), (-/-) × (+/-) breeding pairs were used to generate all offspring. Female (-/-) mice were not fertile due to an apparent susceptibility to genital infections (Dan Pennington, personal communication). All mice used in Chapter 6 were generated from (-/-) male × (+/-) female breeding pairs. The genotypes were not determined until all physiological data had been collected, after which they were determined by polymerase chain reaction (PCR).

4.1.2 Genotyping by polymerase chain reaction

4.1.2.1 DNA isolation and purification

1cm sections of tail were cut from mice (which were identified by ear clipping) and placed in tail buffer (50mM Tris (pH 8.0), 100mM EDTA, 0.5% (w/v) SDS) containing 40µl of proteinase K (10mg/ml) overnight at 55°C in a compact thermomixer (Eppendorf, UK). The solution was then mixed, with shaking for 5 minutes, with 700µl of phenol (equilibrated with Tris pH 8.0). The tubes were then microfuged and 600µl of the aqueous phase transferred to fresh tubes containing 200µl tail buffer. 700µl phenol:chloroform (1:1) was added to each tube, which was subsequently shaken for 5 minutes and microcentrifuged for 5 minutes. Exactly 630µl was then removed from the aqueous phase and added to prepared tubes containing 70µl of 3M sodium acetate (pH 6.0 – a sodium acetate solution of pH lower than 6

will cause the EDTA to precipitate). The DNA was immediately precipitated by the addition of 700µl of 100% ethanol with shaking by inversion. The DNA pellet is recovered by centrifugation and aspiration of the supernatant. Addition of 1ml of 70% (v/v) ethanol and further centrifugation removes the excess salt and the DNA pellet was air dried before resuspension in 80µl DI H_2O.

4.1.2.2 Polymerase chain reaction

The polymerase chain reaction (PCR) reaction mix was prepared by addition of all components shown in Table 2-1, except the DNA, to 200µl PCR tubes. Care was taken to keep all solutions on ice to prevent primer dimerisation. The DNA was quickly added to each tube and the solutions overlaid with mineral oil. The replication reaction was started by addition of 0.5µl Taq polymerase (Promega, 100U/20µl) and the tubes placed in a thermocycler (Techne, UK) preset to 80°C. The machine was held at 80°C for 2 minutes 45 seconds before undergoing 32 cycles at 94°C (1 minute), 60°C (30 seconds), and 72°C (30 seconds). The reaction mixture was then stored at 4°C prior to agarose gel electrophoresis to identify DNA products.

Constituents	**Amount per tube (µl)**
Appropriate reaction buffer (as per manufacturer's instructions)	5
$MgCl_2$	5
dNTP's mix (10%, v/v)	1
primers	0.5 of each (3 primers)
DNA	2
DI H_2O	35.5

Table 2-1: PCR reaction mixture.

4.1.2.3 Agarose gel electrophoresis

25µl of PCR reaction mixture was then added to 5µl of 6× gel loading buffer prior to loading and electrophoresis on preformed 1% (w/v) agarose gels, stained with 0.5µg/ml ethidium bromide. The gel was then visualised using a UV dual-intensity transilluminator (UVP, Genetic Research Instrumentation Ltd., Essex, UK) and photographed with a Polaroid DS34 instant camera with a EP H-7 hood (Polaroid UK Ltd., Hertfordshire, UK).

4.2 The principles of retrograde perfusion

The first preparation capable of maintaining the isolated heart was described by Oscar Langendorff (228). In the Langendorff preparation the ascending aorta is tied to a cannula through which perfusate enters the aortic root. As long as the aortic valve is intact and the pressure inside the left ventricle is less than that in the aortic root, the perfusate is forced through the right and left coronary arteries situated in the coronary sinuses. After passing through the coronary arteries and myocardial capillaries, perfusate drains into the right atrium and ventricle and exits through the pulmonary artery. Providing the perfusate contains essential substrates and the flow is sufficiently high to maintain supply and remove metabolites, the heart can remain viable and contract for many hours.

4.3 Heart isolation and cannulation

All male animals were anaesthetized with a ketamine/xylazine mixture (150mg/kg and 24mg/kg with 100IU heparin, i.p.). Hearts were rapidly excised from the thorax and placed in ice-cold modified Krebs-Henseleit (K-H) buffer. After removal of excess thymic and fatty tissue, the aorta was cannulated with a 23G blunt and grooved stainless steel needle. The heart was retrogradely perfused with K-H buffer at a constant pressure of 80mmHg (108cm H_2O). The K-H buffer was pre-filtered using a 0.8µM micro-filter (Whatman, UK) and constantly gassed with 95% 0_2/5% CO_2.

4.4 Retrograde heart perfusion

Following isolation the heart temperature was monitored continuously by a K-Type thermocouple, passed through the right ventricular wall into the right ventricle, and attached to a C9001 Thermometer (Comark, UK). The temperature was maintained at 37.0±0.1°C by immersing the heart and cannula in K-H buffer kept at 37.0°C in a water-jacketed chamber. A fluid-filled balloon inserted into the left ventricle was used to assess contractile function. The balloon was attached to a pressure transducer, which was coupled to a 4S Powerlab (AD Instruments, UK). The frequency response characteristics of the isovolumic fluid-filled balloons, coupling tubing and transducer were flat to at least 30Hz. The balloon was gradually inflated until the end-diastolic pressure reached between 1 and 5mmHg. Hearts were then paced at 580bpm by a 0.075mm silver wire (Advent, UK) placed through the right ventricular wall into the right ventricular apex. The wire and aortic cannula were attached to an SD9 stimulator (Grass Instruments, USA) delivering square wave pulses of 5mS duration at 1V amplitude.

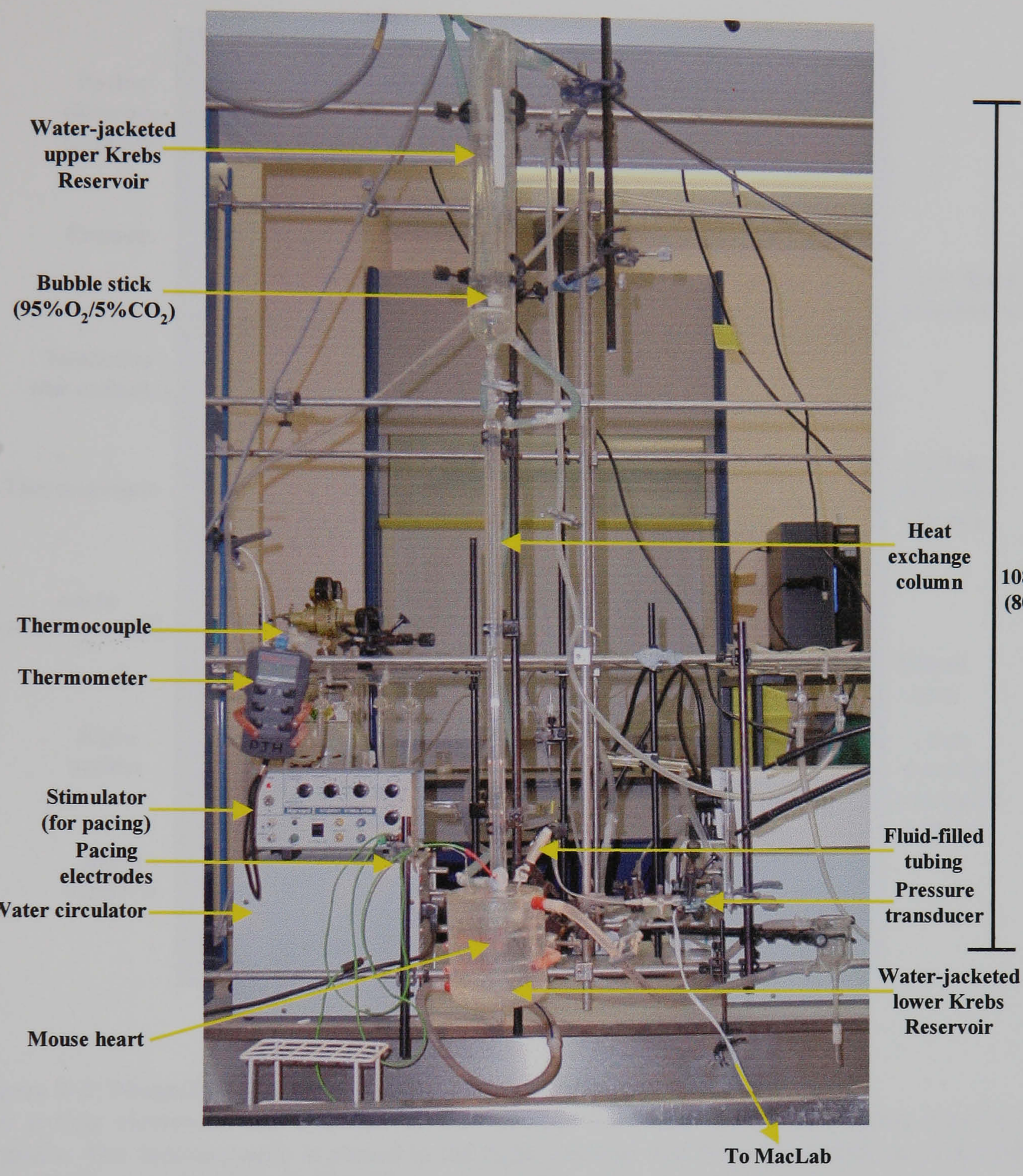

Figure 2-1: Langendorff perfusion apparatus used in studies described within this thesis.
The heart is perfused by oxygenated K-H buffer maintained at 37°C (by heat-exchange column), at a constant pressure of 80mmHg (determined by the height of the upper Kreb's reservoir). The temperature of the heart and inflow cannula is also maintained at 37°C by submersion in the lower Kreb's reservoir. The heart temperature only varied ±0.1°C, as measured by a thermocouple inserted into the right ventricle and attached to an electronic thermometer. The heart is paced at 580 bpm by electrodes attached to a stimulator. The contractile function is monitored throughout via a fluid-filled balloon in the left ventricle attached, though fluid filled tubing, to an online pressure transducer. The output from the transducer is amplified in a bridge-amplifier, converted from analogue to digital and visualised with a MacLab Chart software program (see Figure 2-3).

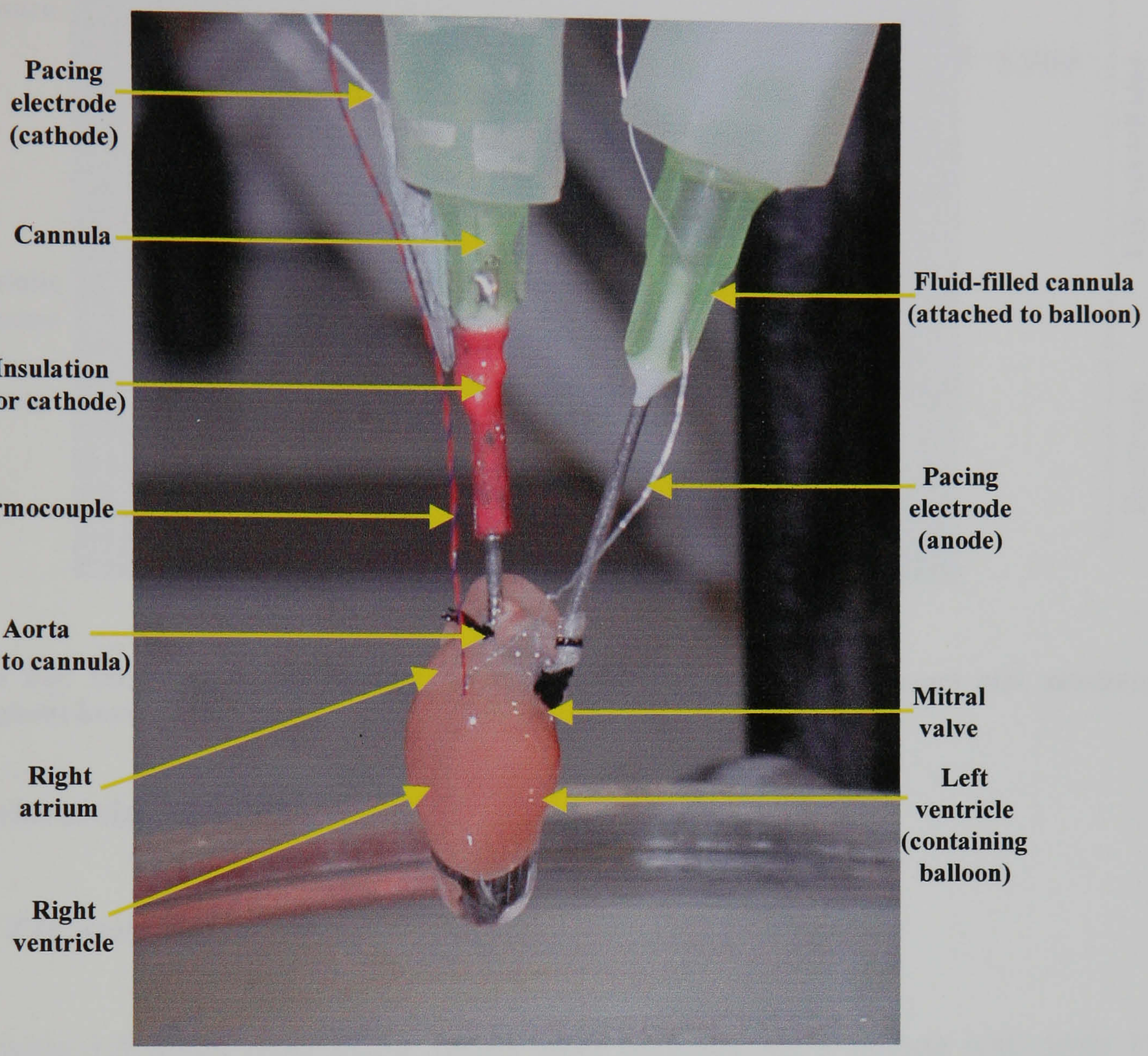

Figure 2-2: Magnified image of a perfused mouse heart.
The pacing electrodes are attached to the cannula (insulated from surrounding Kreb's) and right ventricle. The thermocouple is placed in the right ventricle and fluid-filled balloon in the left ventricle (through the mitral valve). K-H buffer is constantly perfused through the cannula into the aortic root, where it is forced through the left and right coronary arteries.

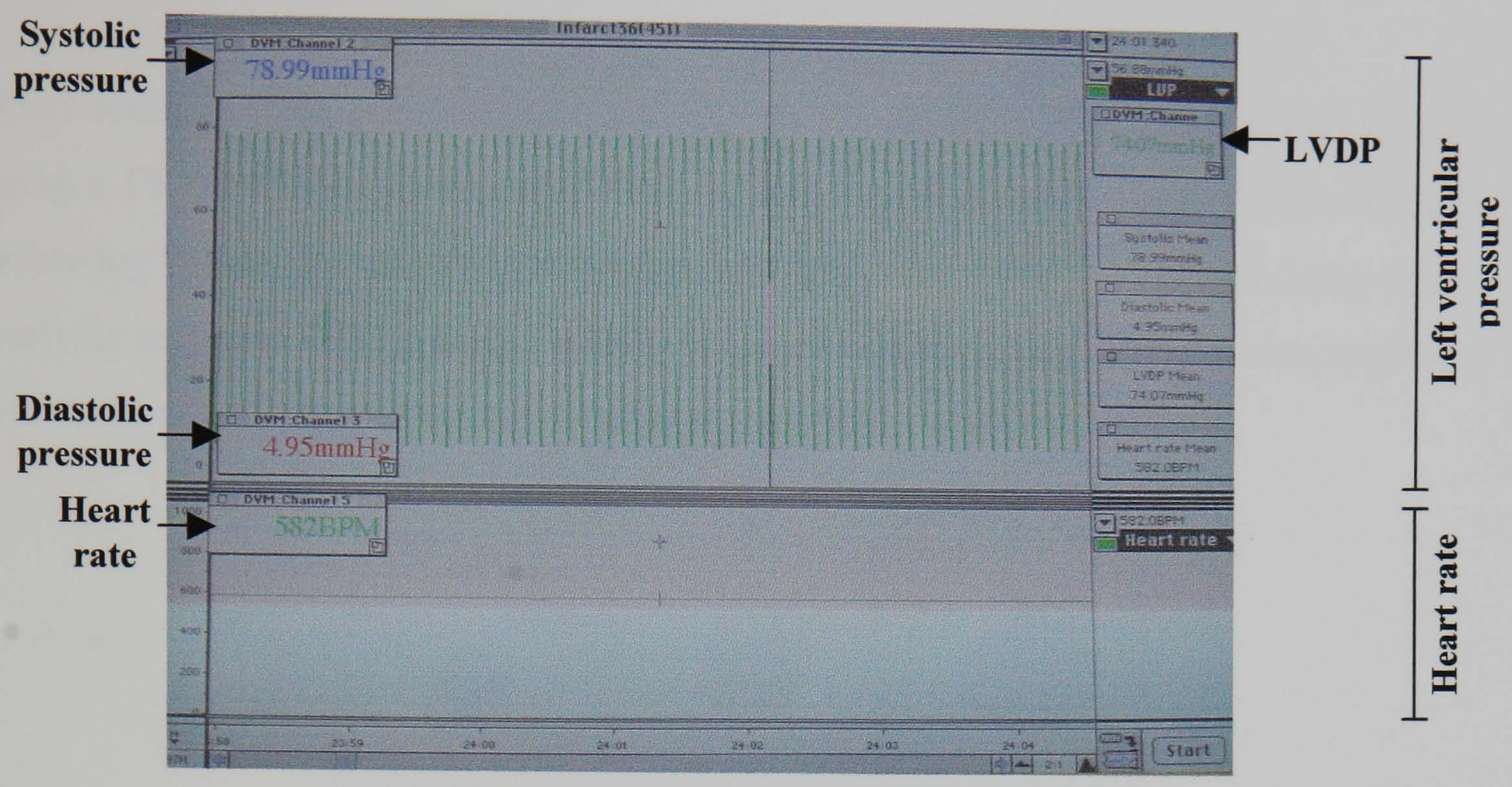

Figure 2-3: Image of digital readouts of left ventricular pressure and heart rate monitored throughout heart perfusion.

4.5 Infarct size assessment

4.5.1 TTC staining

Following 1.5 hours reperfusion, hearts were perfused for 1 minute with 5mls 1% (w/v) triphenyl-tetrazolium chloride (TTC) in phosphate buffer (Na_2HPO_4 45.1mM, NaH_2PO_4 3.3mM, pH 7.8). Hearts were then removed from the cannula and placed in an identical TTC solution at 37°C for 10 minutes. The atria were then removed, and the hearts blotted dry, weighed and stored at –20°C for up to 1 week.

4.5.2 Sectioning and fixation of hearts

Hearts were thawed, placed in 2.5% (v/v) glutaraldehyde for 1 minute and then set in 5% (w/v) agarose solution. The agarose heart blocks were then sectioned from apex to base in 0.7mm slices using a vibratome (Series 1000, Agar Scientific, UK). Following sectioning, slices were placed overnight in 10% (v/v) formaldehyde at room temperature, before transferring into PBS for a further 2 days at 4°C.

4.5.3 Infarct size measurement

Heart sections were compressed between Perspex plates (0.57mm apart) and imaged using a TK-1280E digital camera (JVC, Japan). A typical heart, stained and sectioned following ischaemia, is shown in Figure 2-4. Planimetry was carried out using image analysis software (Scion Image v1.61, Scion corporation, http://www.scioncorp.com) and surface area transformed to volume by multiplication by 0.57.

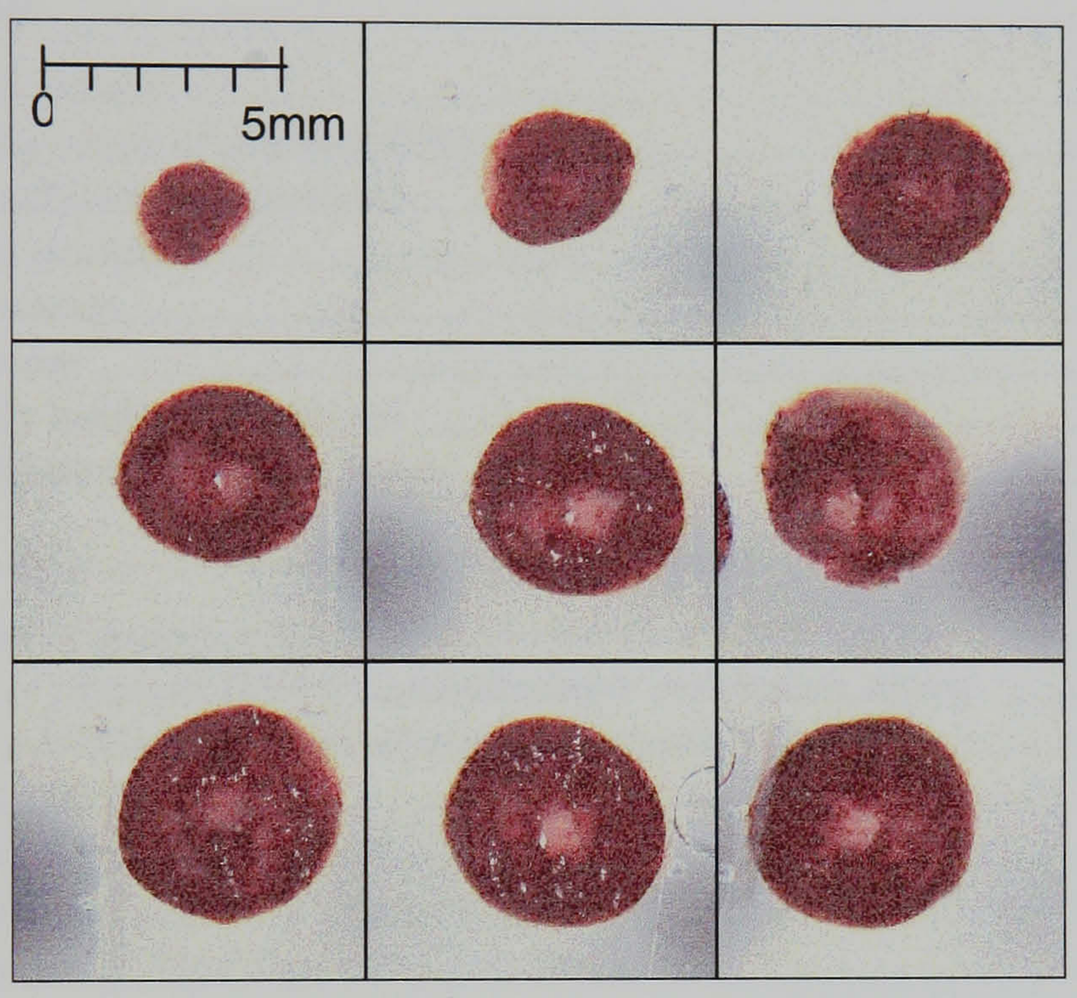

Figure 2-4: Typical mouse heart, subjected to 45 minutes ischaemia, stained with triphenyl tetrazolium chloride (TTC) and sectioned using a vibratome before imaging.

5 STATISTICAL METHODS

All values are expressed as mean±SEM. All comparisons involving more than one group were assessed for significance using one-way analysis of variance (ANOVA), followed where appropriate by the Tukey-Kramer test for pair-wise comparisons. Comparisons between paired samples were performed using one-way analysis of co-variance (ANCOVA). Statistical tests were performed using commercially available software (Sigma Stat v2.03, SPSS Inc., USA). Unless otherwise stated, a statistical value of less than 0.05 was considered significant.

Chapter 3. Activation of PKC isoforms during preconditioning in isolated neonatal cardiomyocytes

1 INTRODUCTION

The binding of membrane phospholipids such as DAG and PS is an essential step in the activation of classical and novel PKCs, since it causes a conformational change that removes the inhibitory pseudosubstrate domain from the catalytic site, thus activating PKC (72) (Figure 1-3, page 40). Previous studies in our lab have exploited these features of PKC activation to engineer mutant PKC isoforms, rendered constitutively active by a deletion within their pseudosubstrate domain (Figure 3-1). Using this approach, we have shown that expression of an active PKCδ mutant is capable of protecting neonatal rat ventricular cardiocytes during simulated ischaemia (221).

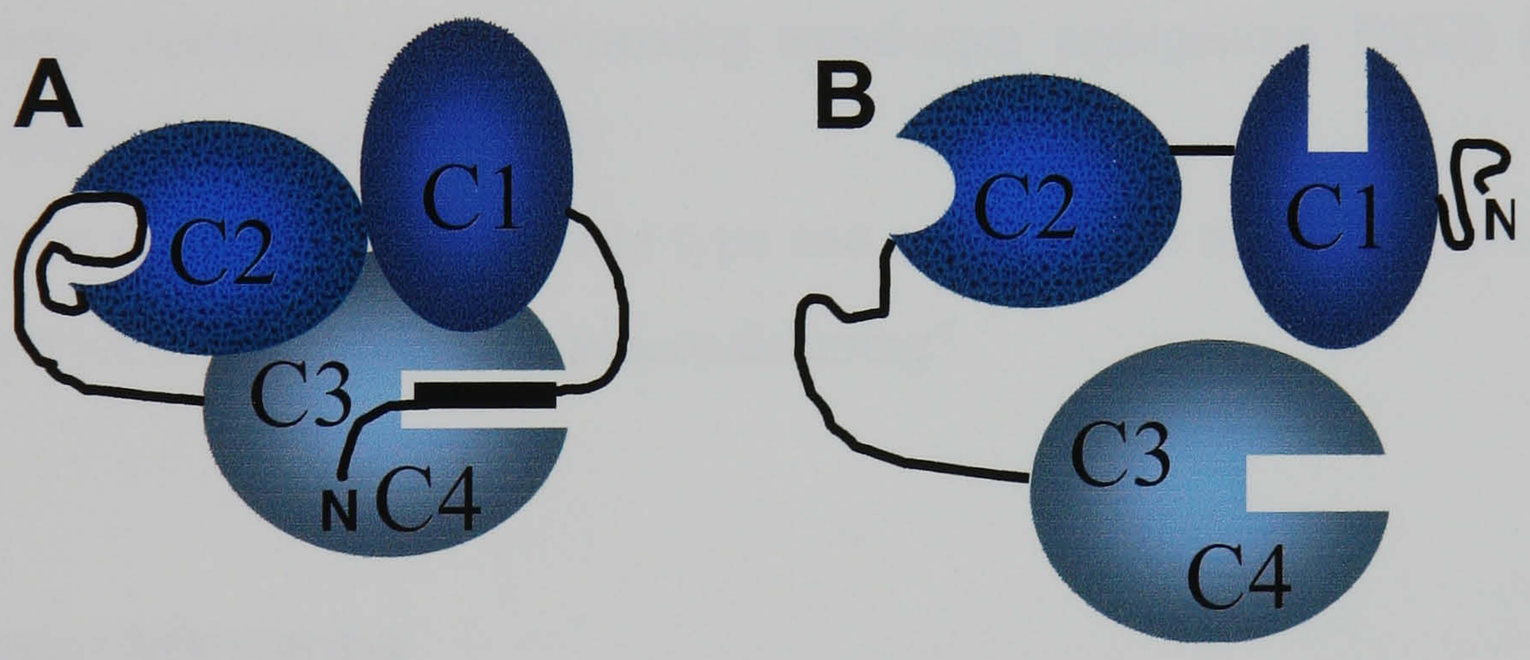

Figure 3-1: Representation of mutant PKC isoforms activated by a pseudosubstrate domain deletion.
The N-terminal pseudosubstrate domain of PKC binds to the catalytic (C4) domain preventing activity in native PKC (*Panel A*). Upon activation, the pseudosubstrate is removed, which enables downstream substrate phosphorylation. A 9a.a. deletion within this pseudosubstrate domain (residues 151-160) prevents its binding to the catalytic domain, thus enabling constitutive substrate activation (*Panel B*).

The protection observed following PKCδ activation, although consistent with other studies (281), does not implicate this isoform in ischaemic preconditioning. Using isoform translocation as a surrogate for PKC activation however, other studies have associated preconditioning-induced protection with the activation of various PKC isoforms, including PKCδ (100, 282-284).

A possible disadvantage of expressing an active PKCδ mutant is that, whilst endogenous PKC needs to be localised to the membrane for activation, mutant PKC does not, since it is active even in the absence of DAG and PS. This raises the question of whether 'constitutively active' PKC exhibits the same spatial localisation as physiologically activated PKC. On the other hand, in the active conformation mutant PKC has the RACK binding domain (within the C2 domain) exposed and therefore maybe constitutively present at the membrane even in the absence of stimulation.

In an attempt to address the aforementioned issues and to corroborate the protection achieved by active PKCδ expression and ischaemic preconditioning, we wished to address the following two questions:

- Does ischaemic preconditioning translocate endogenous PKCδ in isolated neonatal cardiac myocytes?
- Does the localisation of wild type and active PKCδ reflect to the localisation of PKCδ before and after preconditioning?

2 Specific Methods

2.1 HEK 293 cell culture

HEK 293 cells were cultured to confluency in 15mls FGM in a 75cm^2 flask at 37°C. Media was then aspirated, 2mls trypsin (5%, v/v, in versene) added and the flask returned to the incubator for 5 minutes, after which 10mls of FGM was added. 2mls of cell suspension was then added to each well of a 6-well plate and the cells cultured at 37°C for at least 1 day before use.

2.2 Restriction digestion of plasmid DNA

Restriction endonuclease enzymes cut DNA at specific codon sequences. These enzymes were used in restriction digests to verify the PKC isoforms inserted in the

expression plasmid pCAGGS. 1μg DNA, diluted to 17μl in DI H_2O, was added to 1μl restriction enzyme and 2μl of appropriate 10× reaction buffer (as per manufacturers specifications). The mixture was then placed at 37°C for 1 hour, before addition of 4μl of 6 × gel loading buffer prior to electrophoresis on a 1% agarose gel.

2.3 Liposome-mediated transfection

DC-chol: DOPE liposomes were prepared by Louisa Stewart at Imperial College of Science, Technology and Medicine. The liposomes (1.2μg/μl) were mixed with DNA in a 3:1 ratio. The lipoplexes were complexed at 4°C for 30 minutes prior to addition of 1ml SFM. The media containing the complex was then added to HEK 293 cells grown to 80% confluency in a 6-well plate (1ml media/well). The cells were then returned to the incubator for 1 hour before addition if 1ml FGM, after which the cells were incubated for a further 48 hours before harvesting for Western blotting with anti-PKCδ antibodies to detect PKC levels.

2.4 Experimental protocols

The experimental protocols are shown below (Figure 3-2). The end of re-oxygenation was chosen to assay PKC localisation because PKC translocation has previously been documented at this time following preconditioning (284).

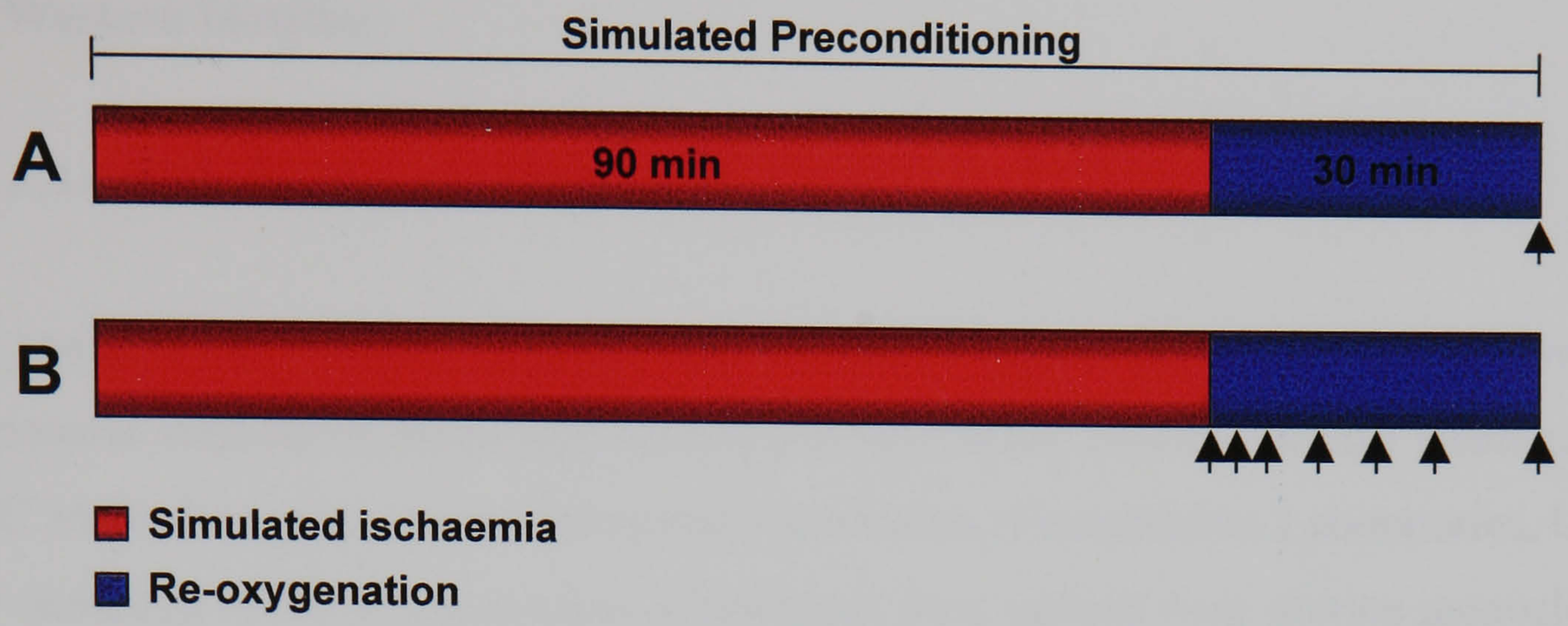

Figure 3-2: Schematical representation of experimental protocols.
Preconditioning was simulated by exposure to ischaemia buffer for 90 minutes followed by 30 minutes re-oxygenation in standard maintenance media. The arrows indicate the time-points at which the cells were harvested and protein samples prepared. ***Panel A,*** PKC localisation was determined at the end of re-oxygenation. ***Panel B,*** MAPK activation was assessed at various time-points during re-oxygenation (0, 2, 5, 10, 15, 20, and 30 minutes).

2.5 Cell Fractionation

Cells were briefly washed 3 times in ice-cold PBS, before addition of ice-cold lysis buffer (100µl/well) containing 0.05% (w/v) digitonin. The extract was incubated at 4°C for 5 minutes with mixing by inversion. A sample was first taken (35µl), containing total cell protein, before the remainder was centrifuged for 2 minutes at 15,000g and 4°C. The supernatant, containing the cytosolic protein, was removed and the pellet resuspended in lysis buffer (35µl) containing 1% (v/v) Triton X-100 and incubate for 5 minutes as before. Further centrifugation at 15,000g, in a microfuge for 15 minutes at 4°C, enabled the separation of particulate (containing triton-soluble membrane proteins, nuclei and filaments) from insoluble material. An equal volume of boiling 2x SB, containing 20% (v/v) mercaptoethanol, was added to each sample before boiling for 5 minutes. 3-4µl of bromophenol blue was then added prior to SDS-PAGE and Western blotting.

2.6 Western blotting

2.6.1 Antibody binding conditions

All samples for Western blotting were run on 10% polyacrylamide gels, following coomassie staining of identically loaded gels to confirm uniform protein loading. All PKC antibodies were murine monoclonal antibodies (Transduction Laboratories, UK). For detection of MAPK activation, membranes were probed were murine monoclonal antibodies specific for ERK2 (Santa Cruz Biotechnology, Santa Cruz, USA), or rabbit polyclonal antibodies specific for p38, phospho-p38 or phospho-ERK1/2 (Cell Signalling, Hitchin, UK). All primary antibodies were incubated at 1:1000 overnight at 4°C, prior to incubation with either peroxidase-conjugated rabbit anti-mouse IgG or peroxidase-conjugated swine anti-rabbit IgG secondary antibodies (DAKO A/S, Denmark) at 1:2500 dilutions for 2 hours at room temperature.

2.6.2 Quantification of protein levels

Densitometry of protein bands were calculated using Scion Image v1.61 (Scion corporation, http://www.scioncorp.com). The density of each band was obtained from the average of three separate readings. The change in phosphorylation was calculated as a percentage of baseline control samples on the same membrane.

3 RESULTS

3.1 Translocation of endogenous PKC isoforms during preconditioning

The PKC activator phorbol 12-myristate 13-acetate (PMA) activates PKC by binding to the C1 domain and anchoring PKC to the plasma membrane. PMA was therefore used as a positive control for PKCδ translocation, since it causes the relocalisation of all C1 domain-containing isoforms (conventional and novel PKCs) from cytosol to particulate fraction. Myocytes were exposed to 80nm PMA for 10 minutes prior to

harvesting for fractionation and Western blotting. Figure 3-3 shows that, whilst in control myocytes PKCδ is situated in the cytosol and particulate fraction, after treatment with PMA, PKCδ is localised almost exclusively in the particulate fraction.

Figure 3-3: The effect of phorbol 12-myristate 13-acetate (PMA) on subcellular distribution of PKCδ.
80nm PMA was added to cell culture media for 10 minutes (+) before the cells were harvested. Constituent protein was then fractionated into cytosol (C) and particulate (P) fractions prior to Western blotting with anti-PKCδ antibodies. Compared to untreated controls (-), PMA caused a rapid translocation of PKCδ from the cytosol to the particulate fraction, although total PKCδ levels (T) remained unchanged.

Having verified the viability of the subcellular fractionation protocol, the location of PKCδ was examined after simulated ischaemic preconditioning. Myocytes were preconditioned by 90 minutes simulated ischaemia and 30 minutes re-oxygenation, prior to harvesting protein for fractionation and Western blotting. Figure 3-4 shows that, similar to PMA treatment, preconditioning causes a redistribution of PKCδ from cytosol to the triton-soluble particulate fraction.

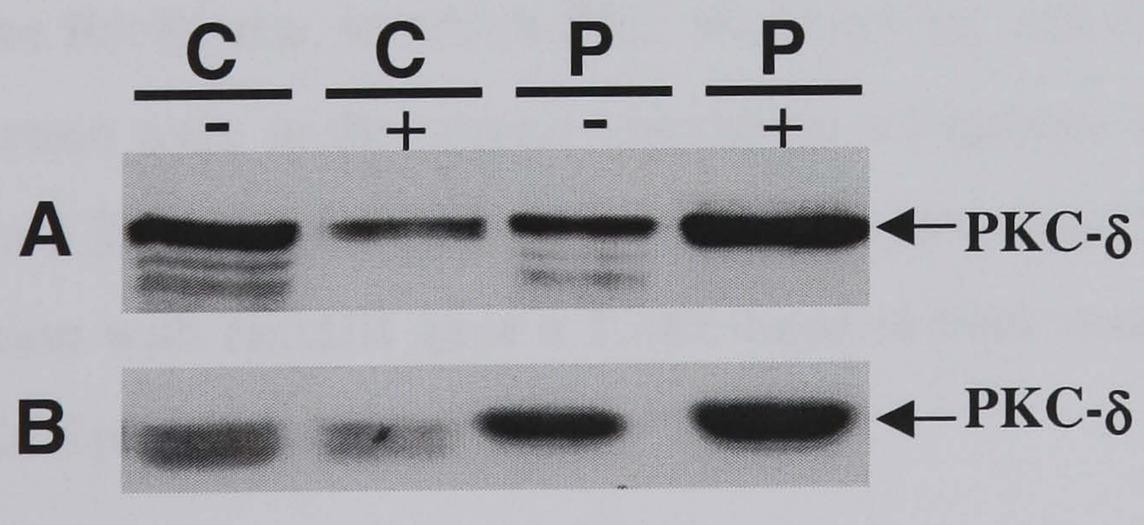

Figure 3-4: The effect of preconditioning on the intracellular localisation of PKCδ.
Myocytes were preconditioned with 90 minutes simulated ischaemia and 30 minutes re-oxygenation (+) before total cell protein was fractionated into cytosolic (C) and particulate (P) fractions. Western blotting with anti-PKCδ antibodies, in two independent experiments (*Panels A and B*), showed that PKCδ is preferentially localised to the particulate fraction following preconditioning

If relocalisation of endogenous PKCδ is responsible for its activation and contributes to the subsequent protection, then ectopically expressed active PKCδ, which confers a similar level of protection to preconditioning, should exhibit this preferential localisation in the absence of preconditioning.

3.2 Verification of plasmid DNA by endonuclease restriction digest

PKC mutants, donated as a kind gift from PJ Parker, are encoded within the multiple cloning site (MCS) of a high efficiency expression plasmid (pCAGGS) (285). Prior to use, the appropriate expression plasmids where checked for their respective PKC coding sequences by endonuclease restriction digest. PKCδ cDNA was inserted into the expression plasmid pCAGGS at an EcoRI restriction site within the MCS. pCAGGS expression vector alone, or containing wild type or active PKCδ were digested with EcoRI to check for presence of the insert. Whilst EcoRI produced 2 fragments of 4.5 and 2.9Kb in wild type PKCδ (Figure 3-5, lane 2) and active PKCδ (Figure 3-5, lane 4), only a single fragment of 4.5Kb was found with expression plasmid alone (lane 5). The 4.5Kb fragment is linearised pCAGGS plasmid, whereas the 2.9Kb fragment corresponds to the size of full length PKCδ. To check the orientation of PKCδ cDNA, plasmids were digested with HindIII, which cuts 500 base pairs 3' of the EcoRI site, in which PKC was inserted, and at base pair 1250 in PKCδ. If the insertion were in the correct orientation a fragment of 1.7Kb would be excised, whereas a 2.2Kb fragment would indicate a reverse orientation. As shown in Figure 3-5, digestion with HindIII gave a 1.7Kb band in both wild type (lane 1) and active (lane 3) PKCδ plasmids.

Figure 3-5: Endonuclease restriction digest of pCAGGS-PKC expression plasmids.
pCAGGS vectors containing wild type PKCδ (lanes 1 and 2), constitutively active PKCδ (lanes 3 and 4) or no insert (lane 5) were cut with either HindIII (lanes 1 and 3) or EcoRI (lanes 2,4 and 5). EcoRI excises the whole PKCδ coding sequence (2.9Kb), whereas HindIII cuts outside the MCS and within the PKCδ sequence, thus excising a 1.7Kb band when the PKCδ coding region is in the correct orientation. The 2.9Kb fragment in lanes 2 and 4 indicates the presence of a PKC insert, whereas the 1.7Kb fragments in lanes 1 and 3 confirm the PKC insertion is in the 3'-5' orientation.

3.3 Verification of DNA transfection/protein expression

3.3.1 In HEK 293 cells

Expression of PKC isoforms was first confirmed in HEK 293 cells, since DNA uptake and/or protein expression is more efficient in this cell line than in neonatal cardiac myocytes. 48 hours post-transfection with cationic liposomes, cells were harvested and protein samples Western blotted with anti-PKCδ antibodies. Overexpression of PKCδ can be seen in both wild type and active PKCδ transfected cells compared to the endogenous level of PKCδ, detected in both empty vector transfected and untransfected cells (Figure 3-6).

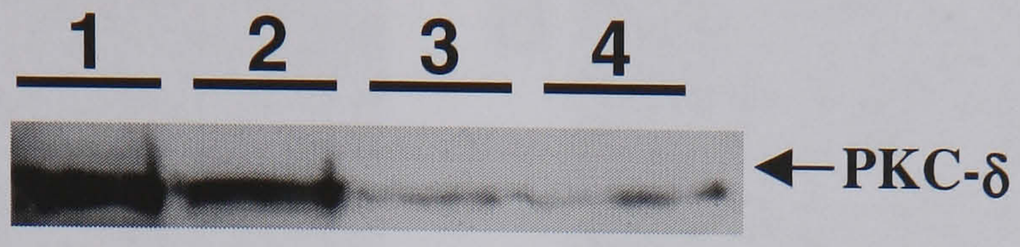

Figure 3-6: Expression of PKCδ plasmids in HEK 293 cells.
HEK 293 cells were harvested 48 hours after transfection with either wild type PKCδ (lane 1), active PKCδ (lane 2), vector alone (3) or vehicle (4). Constituent protein was Western blotted with anti-PKCδ antibodies. A clear overexpression of PKCδ can bee seen following transfection with both wild type and active PKCδ.

3.3.2 In neonatal rat ventricular myocytes

Expression of PKC isoforms in HEK 293 cells does not guarantee expression within neonatal cardiac myocytes, because of differences in transfection efficiency and promoter activity between the two cell types. Initial experiments using a GFP reporter encoded within the pCAGGS plasmid indicated that liposome-mediated transfection was poor, with transfection efficiencies less than 5% (Figure 3-7, *Panel A*). In contrast, integrin-targeting peptides were shown to efficiently infect rat neonatal myocytes, with efficiencies up to 30% (Figure 3-7, *Panel B*). The increased efficiency is probably because integrin peptides utilize a more specific receptor-mediated method of transfection, as opposed to the non-specific endocytosis relied upon by cationic liposomes. Recent evidence however casts doubt over the use of receptor-mediated endocytosis, suggesting instead that the peptide may aid intracellular processing and transport to the nucleus (286). Using the integrin-targeting peptides, overexpression of PKC isoforms could be clearly detected 48 hours post-infection with wild type or active PKCδ (Figure 3-7, *Panel C*).

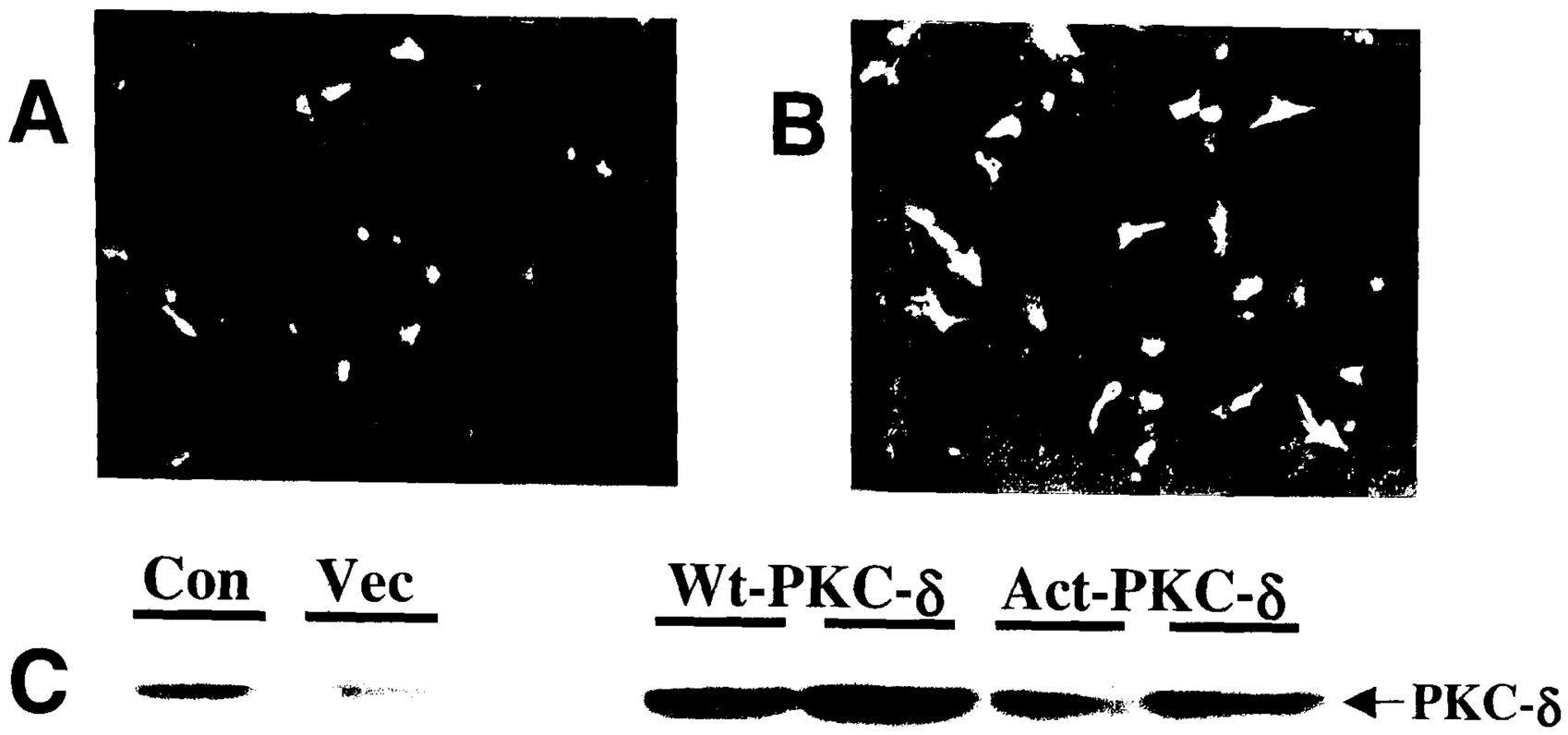

Figure 3-7: Efficiency of transfection in neonatal cardiac myocytes.
48 hours post-transfection with pCAGGS-GFP, myocytes were visualised under UV light to determine expression of the GFP reporter. At his point myocytes were approximately 80% confluent. Liposome-mediated transfection was reproducibly less efficient (*Panel A*) than integrin-targeting peptides (*Panel B*), with efficiencies of approximately 5 and 30% respectively. 48 hours after infection using integrin-targeting peptides cells were harvested and constituent protein probed with anti-PKCδ antibodies (*Panel C*). PKCδ is overexpressed to a similar extent in wild type- and active-PKCδ transfected cells.

3.4 Translocation of ectopically expressed PKCδ

Myocytes were infected with wild type PKCδ or active PKCδ and, 48 hours-post transfection, subjected to 90 minutes simulated ischaemia and 30 minutes re-oxygenation. Thereafter cells were harvested and fractionated as before and Western blotted with anti-PKCδ antibodies. Wild type PKCδ appears equally distributed between cytosol and particulate fraction prior to preconditioning, but is localised mainly in the particulate after preconditioning (Figure 3-8, *Panel A*). This agrees with the findings for endogenous PKCδ seen in untransfected cells (Figure 3-4). Active PKCδ however, shows a similar distribution before preconditioning as seen with wild type PKCδ after preconditioning (Figure 3-8, *Panel B*). This suggests that active PKCδ is similarly localised/activated as endogenous PKCδ following preconditioning, which may represent a key protective signalling event common with both these treatments. After preconditioning in active PKCδ expressing cells, there is a further decrease in cytosolic PKCδ, possible caused by the translocation of endogenous PKCδ.

Figure 3-8: Intracellular localisation of wild type and active PKCδ, prior to and following simulated preconditioning.
Myocytes, infected with wild type (*Panel A*) or constitutively active PKCδ (*Panel B*), were fractionated into cytosolic (C) and particulate (P) fractions, before (-) and after (+) preconditioning. ***Panel A,*** Western blotting with anti-PKCδ antibodies shows that wild type, although equally distributed before preconditioning, is selectively localised in the particulate following preconditioning. ***Panel B,*** however shows that, unlike wild type, active PKCδ is preferentially localised in the particulate fraction even in the absence of preconditioning.

If PKC activation during preconditioning is responsible for subsequent protection, the identification of its downstream substrate(s) may provide new therapeutic targets. One kinase family implicated in preconditioning is the mitogen-activated protein kinases (MAPKs) (126). We therefore sought to examine their activity during preconditioning in isolated neonatal cardiac myocytes.

3.5 Phosphorylation of MAPKs during preconditioning

MAPKs are activated via dual phosphorylation on a Thr-Xxx-Tyr motif (to pThr-Xxx-pTyr). Antibodies specifically raised against this phosphorylated motif allow the direct assessment of MAPK activation. Three members of the MAPK family, p38, c-Jun N-terminal kinases (JNKs) and extracellular signal-regulated kinases (ERKs) were assayed for phosphorylation during an acute preconditioning stimulus in neonatal myocytes. Protein, from cells harvested at various time points during re-oxygenation following 90 minutes simulated ischaemia (as indicated in Figure 3-2), was Western blotted and probed with the appropriate antibodies. No JNK phosphorylation could be detected at any time during re-oxygenation, although antibody affinity was confirmed using a positive control of UV treated cells (results

not shown). Both p38 and ERK1/2 were transiently phosphorylated during re-oxygenation, reaching a maximum after 10 minutes (Figure 3-9).

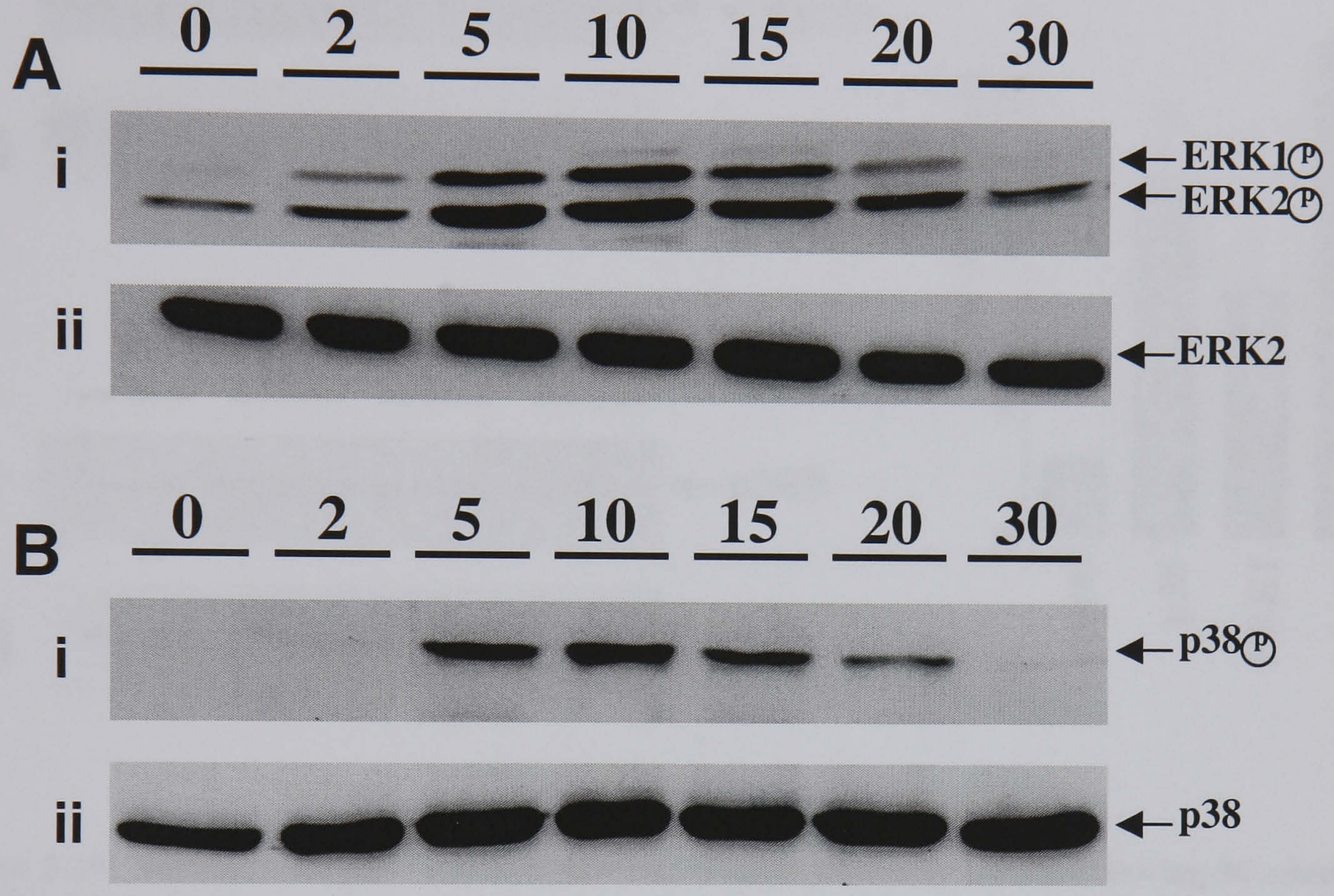

Figure 3-9: Activation of MAPKs during re-oxygenation following 90 minutes simulated ischaemia.
Cells were harvested at various time points (minutes) during the re-oxygenation phase of preconditioning. Constituent protein was probed for phosphorylated ERK1/2 (*Panel Ai*), ERK2 (*Panel Aii*), phosphorylated p38 (*Panel Bi*) or p38 (*Panel Bii*). Both ERK1/2 and p38 become rapidly phosphorylated during re-oxygenation, reaching a maximum at 10 minutes and returning to baseline by 30 minutes. Total p38 and ERK1/2 levels remain constant throughout re-oxygenation.

Having established the time point for maximal MAPK phosphorylation, we harvested cells at 10 minutes re-oxygenation following 90 minutes simulated ischaemia and probed constituent protein for p38 and ERK1/2 phosphorylation to quantify activation. Figure 3-10 shows that p38, ERK1 and ERK2 all exhibit at least a 3-fold activation during the re-oxygenation phase of simulated preconditioning.

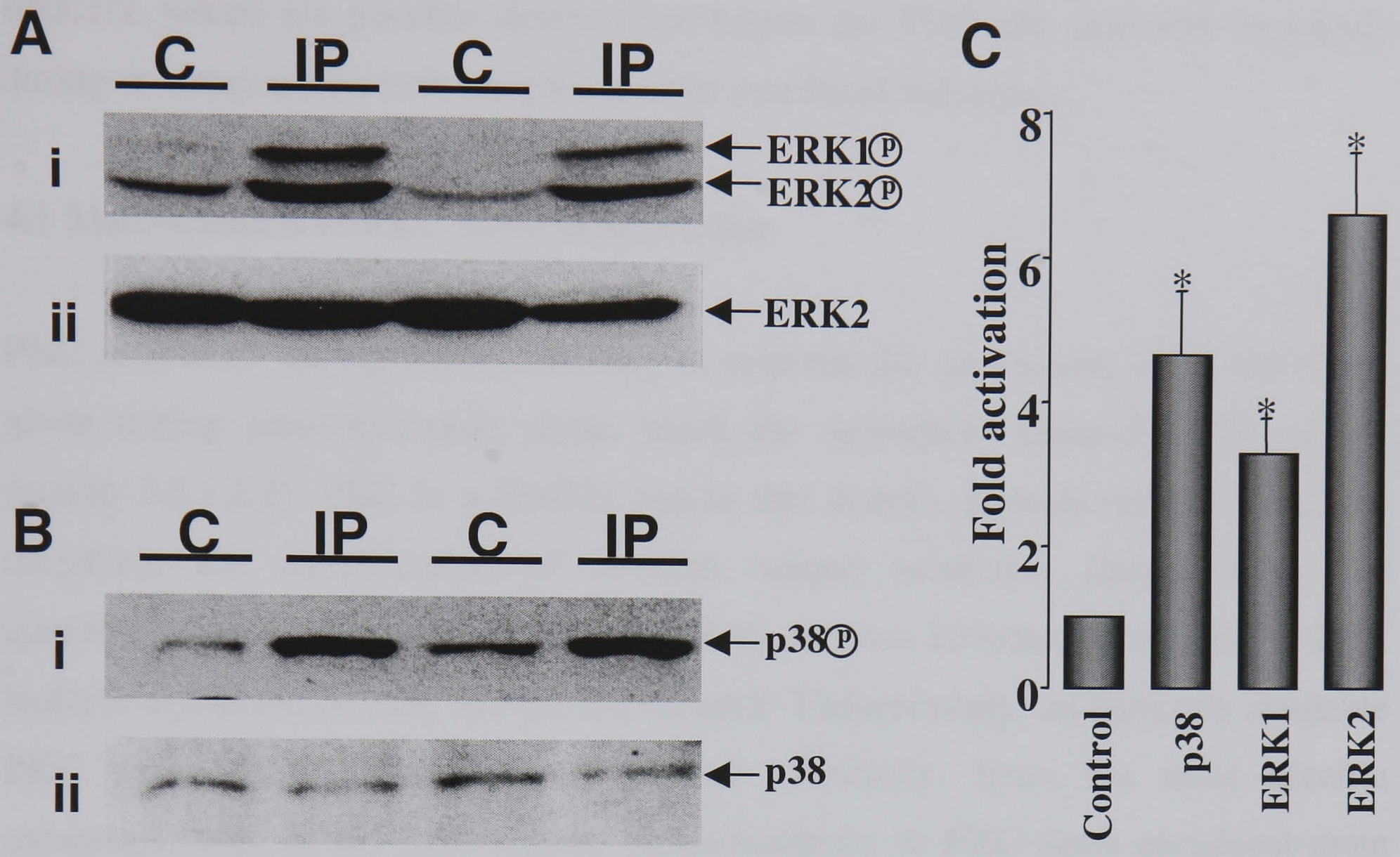

Figure 3-10: Activation of p38-MAPK and ERK1/2 during re-oxygenation following 90 minutes simulated ischaemia.

Myocyte cell lysates were prepared from naïve cells (C) and from myocytes after 90 minutes simulated ischaemia and 10 minutes re-oxygenation (IP). Each pair of samples is from a different cardiocyte preparation exposed independently to simulated ischaemia and re-oxygenation. Samples were probed with dual phospho-specific MAPK antibodies for ERK1/2 (*Panel Ai*) and p38 (*Panel Bi*), or antibodies detecting total ERK2 (*Panel Aii*) and total p38 (*Panel Bii*). ***Panels A and B*** show that both p38 and ERK1/2 are activated during preconditioning. ***Panel C,*** the mean activation of p38, ERK1 and ERK2 during preconditioning as a ratio of that seen in naïve cells. * $p<0.01$ v naïve control, n=5.

4 Discussion

These results demonstrate that PKCδ relocates from the cytosol to the triton-soluble fraction during preconditioning, which may represent activation of this endogenous isoform. Ectopically expressed PKCδ, rendered constitutively active via a deletion within its pseudosubstrate domain, localises preferentially to the triton-soluble fraction under basal conditions. Since this mutant PKC isoform protects against ischaemia it is tempting to suggest that phosphorylation of a substrate(s) in this cellular fraction leads to protection, which may also contribute to protection in

response to preconditioning. The mitogen-activated protein kinases (MAPKs) p38 and ERK1/2, which are possible downstream targets for PKC, are activated transiently during re-oxygenation following 90 minutes simulated ischaemia.

4.1 Measurement of PKC isoform activation

PKC activation during preconditioning is essential for protection, since inhibitors, given during preconditioning alone, block the subsequent protection (Chapter 1, section 3.2.3.2.2). PKC is a Ser/Thr kinase that directly phosphorylates substrates; therefore, the identification of isoform unique substrates should allow the quantification of isoform-specific PKC activity *in vitro*. Demonstrating specific PKC isoform activation directly has proved difficult. Unfortunately, all currently available PKC substrates demonstrate only limited specificity. Even the most specific substrates, such as the PKC-epsilon pseudosubstrate or PKC-alpha pseudosubstrate domains, which are present in the full length PKC isoforms and bind to their respective catalytic sites, are still phosphorylated by other isozymes (287, 288).

PKC isoform activity has been measured using immunoprecipitated PKC isoforms to phosphorylate substrates *in vitro*. Using this approach Ping and coworkers demonstrated activation of PKCε during preconditioning, although the activity of other isoforms was not assessed (289). Similarly, Kitikaze *et al.* utilized the differential requirement for cofactors to implicate a role for conventional PKC isoforms in protection, since preconditioning caused an increase in PKC activity only when calcium was present in the phosphorylation reaction (96). Whilst these techniques can be used to assess changes in activity of individual isoforms, comparisons between isozymes are difficult due to differences in antibody affinity and isotype abundance. Moreover, since this can only be performed *in vitro*, cofactors such as DAG and PS have to be included in the phosphorylation reactions, making extrapolations to cellular levels of activity imperfect. These and other technical limitations in the direct intracellular measurement of PKC isoform activity have prompted preconditioning investigators to use translocation as a surrogate for PKC activation (83).

4.2 Translocation of PKC isoforms during preconditioning

Downey's group first postulated that, "PKC translocation during brief episodes of ischaemia underlies the protective effect of ischaemic preconditioning" (101). This hypothesis has since received substantial support from independent groups (96, 100, 282, 290-292). The activation of PKCδ in neonatal cardiac myocytes is known to protect against simulated ischaemia and re-oxygenation (221). To determine whether endogenous PKCδ was activated during preconditioning in this model, the translocation of this isoform was assessed following simulated ischaemic preconditioning.

4.3 The measurement of PKC localisation

The majority of studies that document translocation of PKC isoforms during preconditioning use subcellular fractionation followed by Western blotting with isoform-specific PKC antibodies (96, 282-284, 293), although immunofluorescence microscopy of heart sections has been used (100, 294). To measure translocation of PKCδ in neonatal cardiac myocytes, cells were first lysed with a mild detergent, digitonin, which is sufficient to disrupt the sarcolemmal membrane and release cytoplasmic proteins into the supernatant (*cytosolic fraction*). The remaining insoluble pellet is then washed with buffer containing 1% (v/v) triton X-100, which solubilises membrane-associated proteins, nuclei and filaments. When PKC is activated and associated with the membrane, through interactions with DAG and PS, it is only soluble in the presence of triton (*particulate fraction*). This is confirmed by PMA treatment, which causes the redistribution of cytosolic PKCδ to the particulate fraction (Figure 3-3). A similar translocation of PKCδ is detected following ischaemic preconditioning, suggesting this isoform is also activated following this treatment (Figure 3-4).

4.4 The localisation of mutant PKC isoforms

A mutant PKCδ, rendered constitutively active by a deletion within its pseudosubstrate domain, is able to protect myocytes against ischaemia (14). A problem levelled at this approach is the uncertain relevance to physiological PKCδ activation. For example, a key step in the activation of endogenous PKC is the relocalisation to the membrane where, upon activation by pseudosubstrate domain removal, it is localised with common downstream substrates. Mutant PKCδ however, due to the absence of an inhibitory pseudosubstrate domain, does not require relocalisation for activation. It is questionable therefore whether mutant PKCδ activates the same membrane-localised substrates. By examining the localisation of ectopically expressed PKCδ, we observed a high level of "active" PKCδ in the membrane prior to preconditioning. This suggests that in the active conformation PKCδ still preferentially relocates from the cytosol to the membrane. This is consistent with the RACK theory for PKC activation proposed by Mochly-Rosen (295). This hypothesis proposes that receptors (termed RACKs), present on the inner surface of the membrane, preferential bind PKC in its active conformation. The selectivity for the active form originates from a RACK binding site in the C2 domain, which is concealed in the inactive closed conformation, but exposed by the conformational change that accompanies PKC activation (see Figure 1-3, page 40). It is likely that this site is already exposed in constitutively active PKCδ, which promotes receptor interaction and membrane localisation in the absence of stimulation.

4.5 Mitogen-activated protein kinase activation during preconditioning

Activation of the mitogen-activated protein kinase (MAPK) family has been reported following preconditioning (124). Moreover, this MAPK phosphorylation has been attributed to prior activation of PKC (223, 296). We therefore wanted to quantify the level of MAPK activation during preconditioning in isolated neonatal cardiac myocytes. No significant phosphorylation of any MAPK family members was seen during 90 minutes simulated ischaemia (results not shown), however transient

phosphorylation of both p38- and ERK1/2-MAPK was seen during the 30 minutes re-oxygenation following ischaemia (Figure 3-9). This activation, which was represented by at least a 3-fold increase in dual phosphorylation, was maximal for p38 and ERK1/2 after 10 minutes re-oxygenation (Figure 3-10).

4.6 Critique of methods

The fractionation method described within this chapter was modified from a protocol previously used to assess PKC translocation during preconditioning is isolated rabbit cardiomyocytes (293). Whilst sufficient for assessing translocation away from the cytosol, this method does not clearly define the exact intracellular location of PKC. PKC does not necessarily need to be localised at the sarcolemmal membrane for activation, since the lipid membranes of subcellular organelles, such as the mitochondria, will be sufficient to activate PKC. Using immunofluorescent microscopy with anti-PKCδ and –PKCε antibodies, Mitchell *et al.* showed that PKCδ translocates to the sarcolemmal membrane and PKCε to the nuclei following brief ischaemia in the rat (100). Using a similar technique, and the same model, Wang *et al.* showed that PKCδ is localised to mitochondria and intercalated discs following diazoxide treatment, which protects against ischaemic injury presumably by the opening of K_{ATP} channels (292), a mechanism that has also been implicated in ischaemic preconditioning (155). A better understanding of PKCδ compartmentalisation during preconditioning may aid the discovery of co-localised downstream substrates. Furthermore, it would be interesting to compare the exact location of constitutively active PKCδ with reference to physiologically activated PKCδ during preconditioning.

As discussed earlier, it is believed that PKC translocation is required for PKC activation, this does not however imply that the translocation of PKC is synonymous with activation. PKC binds to a large number of proteins, most of which display isoform specificity. These interactions probably significantly contribute to the differential effects of various PKC isozymes (297). Proteins that bind PKC are classed according to their preference for activation state. Whilst some of the binding proteins

have been shown to preferentially bind activated PKC (receptors for activated C-kinase or RACKs (104)), others specifically bind, and perhaps stabilise, inactive isoforms (receptors for inactive C-kinase or RICKs (105)), whilst yet other proteins, such as calveolin, interact with PKC and cause inhibition of activity (298). To add further complexity, although some binding proteins act as substrates (78), others serve to cluster signalling molecules by acting as scaffold proteins (78). Therefore, translocation cannot only be associated with activation, but also inactivation of PKC. Ideally, PKCδ should be immunoprecipitated from the particulate fraction following preconditioning and assayed for activity, although the necessary inclusion of DAG and PS in phosphorylation reactions further complicates the interpretation of activity assays.

To unequivocally demonstrate PKCδ translocation following preconditioning, multiple western blots should be performed on independent samples and the band densities quantified by densitometry. This would give an indication of the level of PKC translocation and provide statistical data on PKCδ localisation.

4.7 Conclusions

Following ischaemic preconditioning, and immediately prior to lethal ischaemia in isolated neonatal cardiac myocytes, PKCδ is preferentially localised to the particulate fraction, which may represent increased activity of this novel PKC isoform. It is tempting to suggest that this activation/translocation contributes to protection because expressing a constitutively active PKCδ mutant protects to a similar extent, and cause a similar translocation of PKCδ, in the absence of preconditioning. Selective inhibition of this isoform during preconditioning would however be required to confirm its role during preconditioning in this model.

Both p38- and ERK1/2-MAPK are activated during preconditioning in this model. The MAPK phosphorylation, seen during re-oxygenation following 90 minutes simulated ischaemia, maybe a direct or indirect consequence of PKC activation. It would be interesting to determine the time-course of PKC translocation during

preconditioning to see if this correlates with the time of MAPK phosphorylation. Furthermore, subcellular fractionation of myocytes after 10 minutes re-oxygenation would reveal the proximity of phosphorylated MAPK to PKC.

Chapter 4. MAPK activation during preconditioning and ischaemia

1 INTRODUCTION

In the previous chapter we demonstrated activation of PKCδ, p38-MAPK and ERK1/2-MAPK during preconditioning in neonatal cardiocytes. Previous work in our laboratory has already demonstrated protection in response to PKCδ activation. Therefore the immediate questions to address were:

- Does PKCδ activation protect through activation of MAPKs?
- Does the activation of PKCδ during preconditioning contribute to protection by MAPK activation?

Therefore we examined the interplay between PKC and MAPKs during simulated preconditioning in neonatal cardiocytes

2 SPECIFIC METHODS

2.1 Experimental protocols

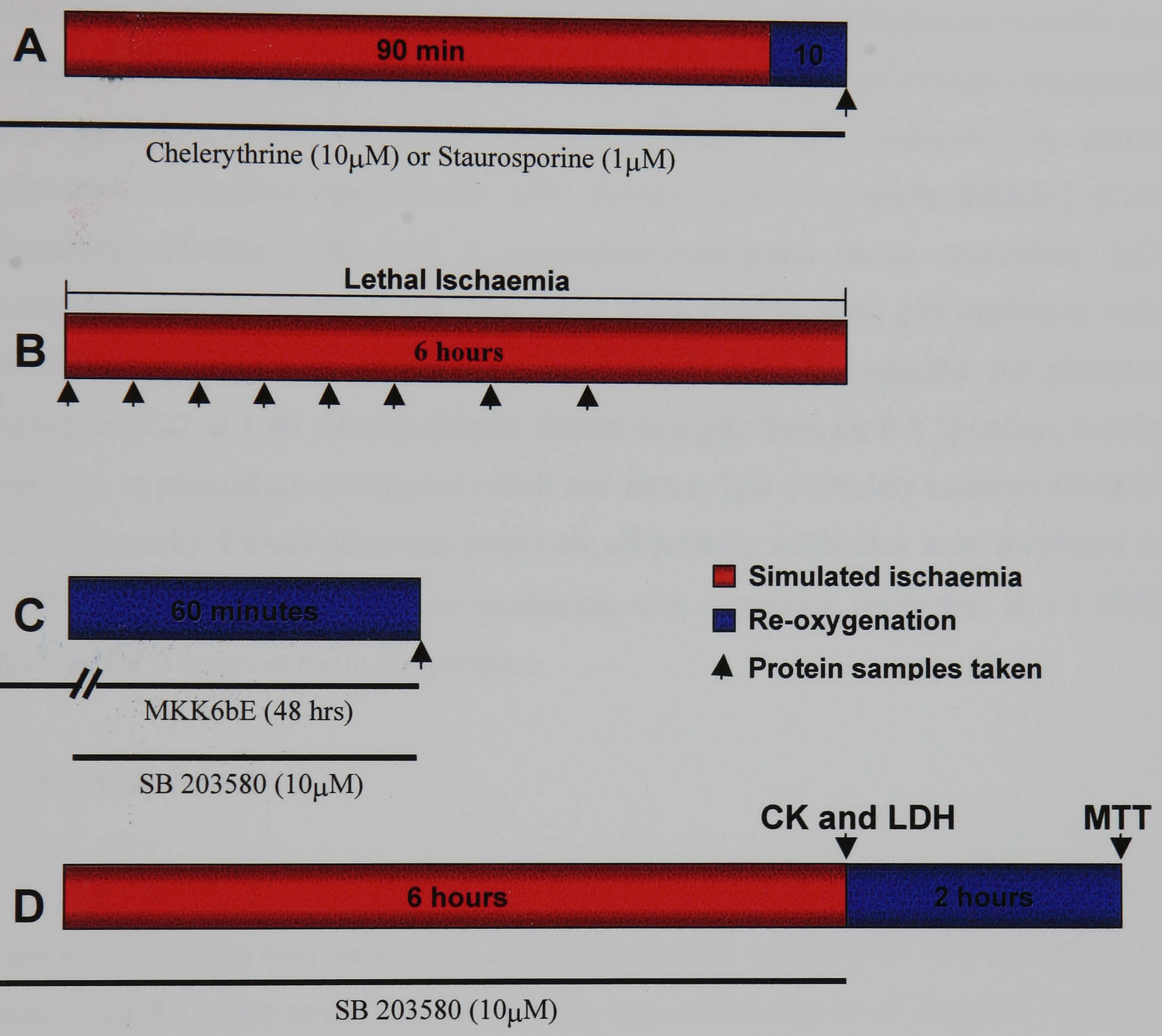

Figure 4-1: Schematical representation of experimental protocols.
Panel A, cells were harvested 10 minutes into re-oxygenation following 90 minutes simulated ischaemia in the presence of chelerythrine (10μM) or staurosporine (1μM), to assess the contribution of PKC to MAPK activation during preconditioning. ***Panel B,*** cells were lysed and protein prepared at various time-points during ischaemia to assess MAPK activation. ***Panel C,*** the inhibition of p38 activity by SB203580 was assayed in cells transfected with constitutively active MKK6b. Cells were incubated with 10μM SB203580 for 1 hour or left untreated, before harvesting and Western blotting of constituent protein with anti-phospho-HSP27 antibodies. ***Panel D,*** the role of p38 in ischaemic injury was determined by incubation with SB203580 (10μM) throughout ischaemia. Enzyme release was measured immediately after lethal ischaemia and MTT bioreduction after a further 2 hours re-oxygenation.

2.2 Western blotting conditions

All samples for Western blotting were run on 10% polyacrylamide gels, following coomassie staining of identically loaded gels to confirm uniform protein loading. Blots were sequentially probed with either murine monoclonal antibodies specific for ERK2 (Santa Cruz Biotechnology, Santa Cruz, USA) and a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (DAKO A/S, Denmark), or rabbit polyclonal antibodies specific for p38, phospho-p38 or phospho-ERK1/2 (Cell Signalling, Hitchin, UK) and a peroxidase-conjugated swine anti-rabbit IgG secondary antibody (DAKO A/S, Denmark). Blots used to assay p38 inhibition with SB203580 were probed with murine monoclonal antibodies specific for phospho (ser82)-HSP27 at 1:40 dilution (kindly donate as a gift from Dr RA Quinlan), before exposure to peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (DAKO A/S, Denmark). Unless otherwise indicated, all primary antibodies were incubated at 1:1000 overnight at 4°C, prior to incubation with secondary antibodies at a 1:2500 dilution for 2 hours at room temperature.

2.3 Statistical analysis

All values are expressed as mean±SEM. Data for individual treatments were collected from no more than two wells from each experimental preparation. The "n" numbers under "results" relate to the number of wells from which data were obtained. For each treatment mean values were pooled to allow statistical comparisons. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA), followed where appropriate by the Tukey-Kramer test for pair-wise comparisons. All analyses were performed using Statview version 4.0 statistical package (Abacus Concepts Inc., Berkeley, CA). A probability value ≤ 0.05 was considered significant.

3 RESULTS

3.1 Is MAPK activation downstream of PKC?

The specific phosphorylation of MAPKs observed during re-oxygenation (Figure 3-10) may be a consequence of PKC activation, since it has been reported previously that PKC is activated at the onset of reperfusion (291). If this were true in our model, and the isoform involved is PKCδ, then we should observe a comparable level of MAPK phosphorylation in cells overexpressing active PKCδ, even in the absence of preconditioning ischaemia. Therefore to test this hypothesis we transfected myocytes with constitutively active PKCδ, in an attempt to mimic the pattern of p38 and ERK1/2 phosphorylation seen following 90 minutes simulated ischaemia and 10 minutes re-oxygenation.

3.1.1 Effect of active PKC expression on MAPK activation

Myocytes were transfected with the eukaryotic expression plasmid pCAGGS encoding either wild type or active PKCδ. After 48 hours, myocytes were either harvested, to assess basal MAPK phosphorylation, or subjected to 90 minutes simulated ischaemia and 10 minutes re-oxygenation to examine preconditioning-induced MAPK activation. It is apparent from Figure 4-2 that overexpression of active PKCδ has no effect on basal ERK1/2 phosphorylation compared to untreated or empty vector transfected controls (*Panel Ai*). This suggests that PKCδ activation does not lead to ERK1/2 phosphorylation. To our surprise however, preconditioning-induced ERK1/2 phosphorylation is completely abolished in the presence of active PKCδ. To test whether this was an effect on ERK1/2 expression levels or a selective effect on phosphorylation, identical samples were probed with an ERK2 antibody non-selective for active or inactive forms. The total levels of ERK2 between treatments remain unchanged, indicating the decreased activation is not due to a downregulation of MAPK, but rather an effect on its phosphorylation (*Panel Aii*). Looking at p38 activation in the same samples we observed a similar effect where, although p38 exhibits a higher basal activation in cells expressing active PKCδ, once

again the increase in p38 phosphorylation associated with preconditioning is completely abolished (see Figure 4-2: *Panel Bi*).

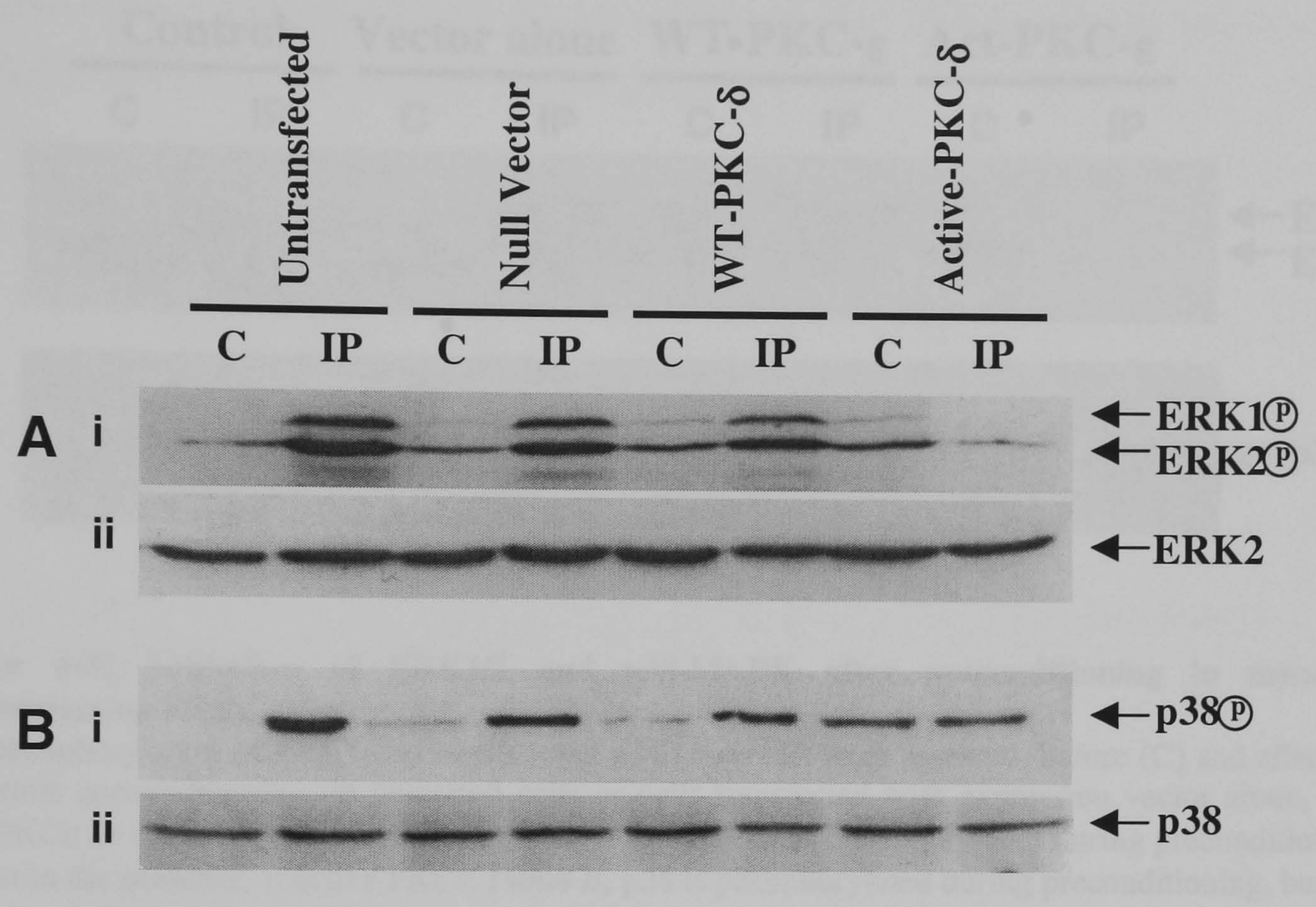

Figure 4-2: Activation of ERK1/2 and p38-MAPK after preconditioning in myocytes overexpressing PKCδ.
Western blots probed with anti-phospho-ERK1/2 (*Panel Ai*), anti-ERK2 (*Panel Aii*), anti-phospho-p38 (*Panel Bi*), or anti-p38 (*Panel Bii*) antibodies. Constituent proteins in both panels were derived from the same experimental groups. Naïve cells (C) were compared to preconditioned (IP) in untreated cardiocytes, cells transfected with expression plasmid containing an empty multiple cloning site (null vector), encoding wild type PKCδ (WT-PKCδ) or constitutively active PKCδ (Active-PKCδ). ***Panel A***, maximal ERK1/2-MAPK activation was assessed, in cell lysates harvested after 10 minutes re-oxygenation following 90 minutes simulated ischaemia, using dual phospho-specific ERK1/2 antibodies (i). Total ERK2 levels were detected using a monoclonal anti-ERK2 antibody (ii). ***Panel B***, p38 activation was detected using phospho-specific p38 antibodies (i) and total p38 was detected using anti-p38 antibodies (ii). For both p38 and ERK1/2-MAPK, the increase in phosphorylation induced by preconditioning is abolished by active PKCδ expression.

These results suggested that, rather than activating, PKC may actually inhibit MAPK activation during preconditioning, because increasing PKCδ activity lessens MAPK phosphorylation. To test whether this was an isoform-specific effect, the experiment was repeated using wild type or constitutively active PKCε, the other PKC isoform implicated in preconditioning (125). Active PKCε decreased both p38 and ERK1/2 activation during preconditioning to a similar extent as seen with PKCδ (Figure 4-3).

In summary, two PKC isoforms implicated in preconditioning are able to inhibit MAPK activation during simulated ischaemia and re-oxygenation.

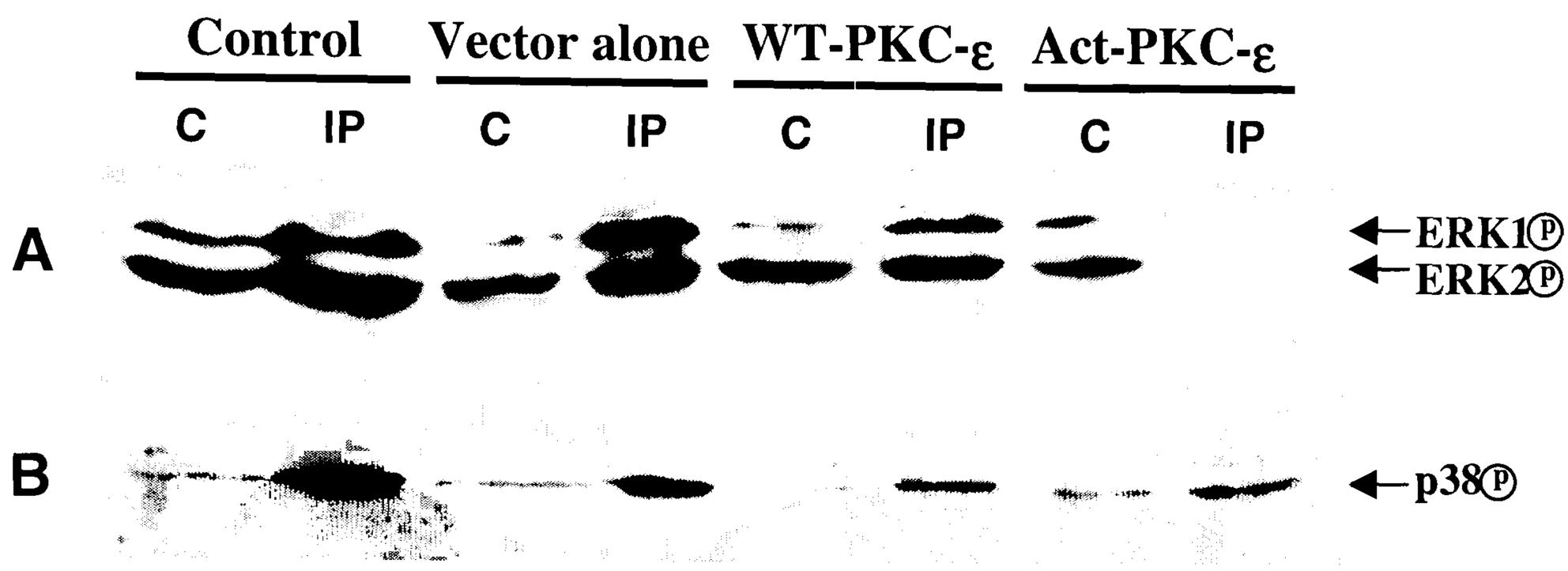

Figure 4-3: Activation of ERK1/2 and p38-MAPK after preconditioning in myocytes overexpressing PKCε.
The phosphorylation of ERK1/2 (*Panel A*) and p38 (*Panel B*) were assessed, before (C) and after (IP) ischaemic preconditioning, in untreated cells or cells transfected with expression vector alone, wild type PKCε or constitutively active PKCε. ***Panel A,*** ERK1/2 is phosphorylated during preconditioning, but not in the presence of active PKCε. ***Panel B,*** p38 is phosphorylated during preconditioning, but this activation is less marked in cells expressing wild type or active PKCε.

It is possible that the effect on MAPK is a consequence of PKC overexpression, which is not observed under physiological conditions. To test this possibility the activation of MAPK was examined during preconditioning in the presence of PKC inhibitors. If PKC were active endogenously during preconditioning then we would expect the activation of MAPK to be limited and thus increased by PKC inhibition.

3.1.2 Effect of PKC inhibition on MAPK activation

Two separate PKC inhibitors were used, chelerythrine and staurosporine (at previously reported concentrations (221)), since they act differently by binding to the regulatory or catalytic domains respectively (Sir P Cohen, personal communication). Inhibitors were given 15 minutes prior to, and throughout preconditioning, before cells were harvested after 10 minutes re-oxygenation and constituent protein subjected to Western blotting to analyse both p38 and ERK1/2 phosphorylation. In the presence of either inhibitor during preconditioning, p38 activation during re-oxygenation was

enhanced, although ERK1/2 phosphorylation was unchanged (Figure 4-4, *Panels Ai and Bi*). Blots probed with antibodies detecting total p38 or ERK1/2 verified this was a specific effect on phosphorylation, as total protein levels remain unchanged (Figure 4-4, *Panels Aii and Bii*). These results confirmed that PKC is able to suppress the activation of p38-MAPK during preconditioning.

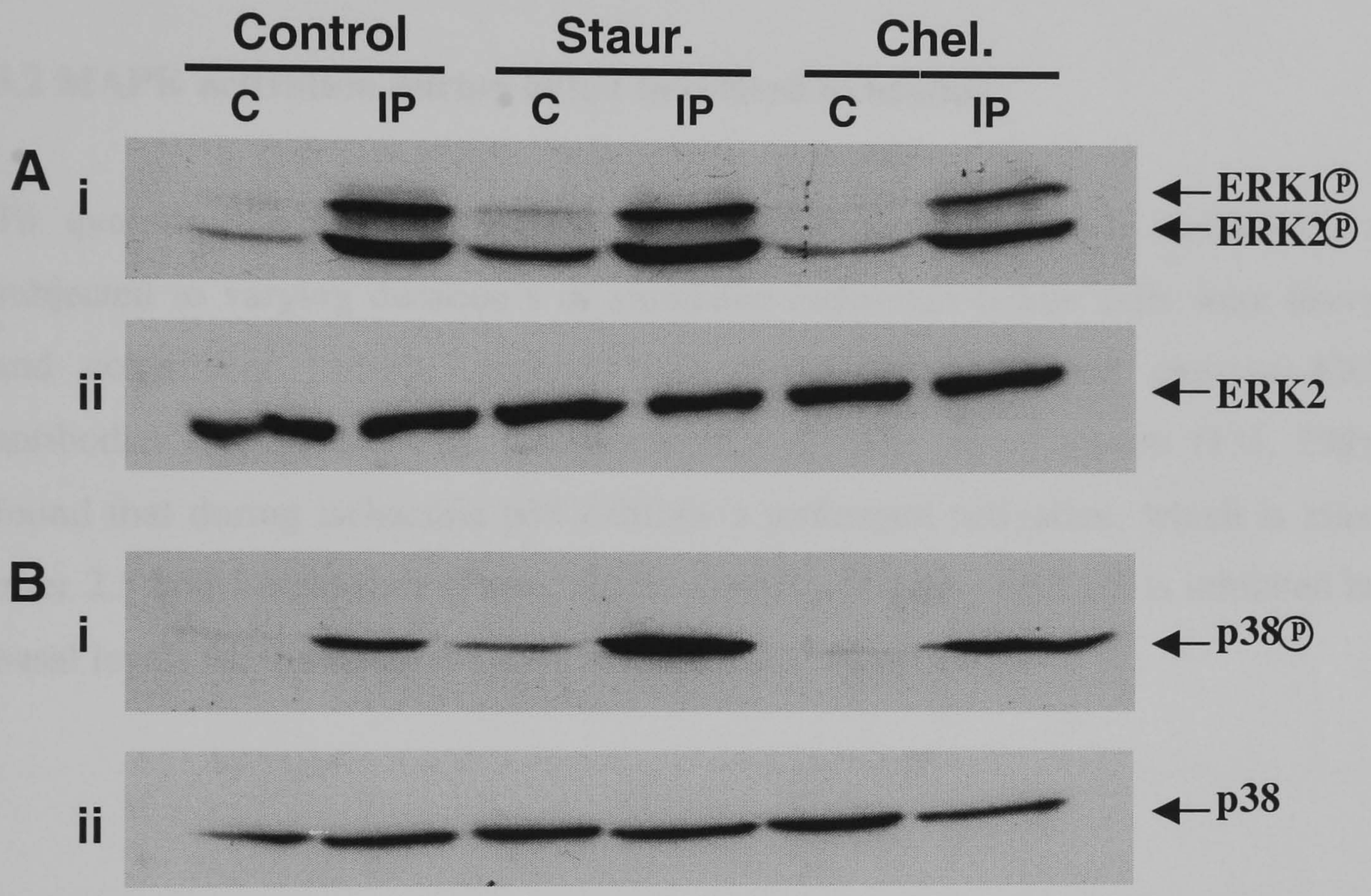

Figure 4-4: ERK1/2 and p38-MAPK activation during preconditioning in the presence of PKC inhibitors.
Myocytes were untreated (C) or preconditioned (IP) in the presence or absence of the PKC inhibitors chelerythrine (10μM) and staurosporine (1μM). After 10 minutes re-oxygenation, ERK1/2 (*Panel A*) and p38 (*Panel B*) phosphorylation was assessed, as before using dual-phospho-specific antibodies. ***Panel A,*** The phosphorylation (i) and total levels (ii) of ERK1/2 are unaltered by PKC inhibition. ***Panel B,*** p38 phosphorylation during preconditioning is enhanced in the presence of either chelerythrine (chel.) or staurosporine (staur.).

These data did not support our original hypothesis that PKCδ protects by causing the same activation of MAPKs that occurs with preconditioning. In contrast, and paradoxically, these data suggest that PKCδ (and possibly other PKC isoforms, including PKCε) inhibits MAPK activation in response to simulated ischaemia/re-oxygenation. It is possible that this unexpected negative regulation may be the

mechanism through with PKCδ protects against lethal ischaemia (221). This alternative hypothesis is consistent with reports examining the role of p38 during ischaemia in the absence of preconditioning, which show that inhibition is protective (131, 133). However for PKCδ to protect via this mechanism the same inhibitory effect on MAPKs must occur during prolonged lethal ischaemia. Therefore we delineated the MAPK pathways that were activated by simulated ischaemia alone.

3.2 MAPK activation during lethal simulated ischaemia

To quantify the level of MAPK activation during ischaemia, cardiocytes were subjected to varying duration's of simulated ischaemia before cells were harvested and constituent proteins probed with anti-phospho-p38 and phospho–ERK1/2 antibodies (see Figure 4-5). In agreement with other recent reports (131, 299), we found that during ischaemia p38 exhibits a prolonged activation, which is maximal after 2.5 hours ischaemia (*Panel B*). In contrast, phospho-ERK1/2 is inhibited below basal levels for the entire duration of ischaemia (*Panel A*).

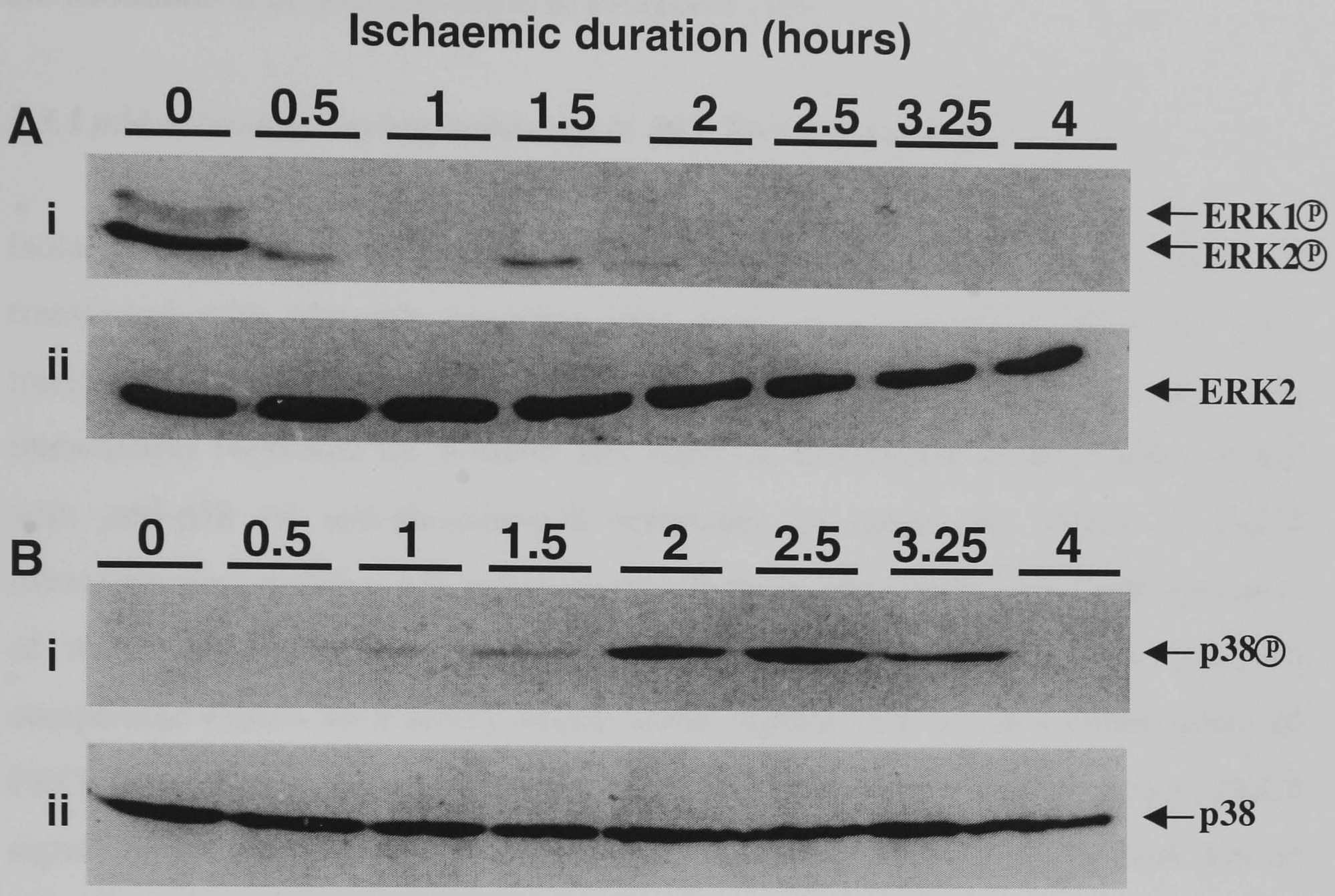

Figure 4-5: Time course of ERK1/2 and p38-MAPK activation during prolonged simulated ischaemia in naïve cardiocytes.
Cardiocytes were subjected to varying duration's of simulated ischaemia (0-4 hours) before cells were lysed for Western blot analysis. Constituent proteins were probed with dual-phospho-specific ERK1/2 antibodies to assess ERK1/2 activation (*Panel Ai*), or anti-ERK2 antibodies to examine total ERK2 levels (*Panel Aii*). Identical samples were also probed with anti-dual-phospho-p38 antibodies to detect p38 activation (*Panel Bi*), or anti-p38 antibodies to assess total p38 levels (*Panel Bii*). ***Panel A,*** ERK1/2 become rapidly dephosphorylated during ischaemia with no change in total ERK levels. ***Panel B,*** p38 is phosphorylated 2 hours into ischaemia and remains activated for a further 2 hours.

This suppression of ERK1/2 during ischaemia eliminates the possibility that PKCδ protects through negative regulation of ERK1/2 phosphorylation. Therefore we wished to examine the result of PKCδ overexpression on the activation of p38 during simulated ischaemia.

3.3 Modulation of p38 activation in protected cells

3.3.1 p38 activation during ischaemia in PKCδ transfected cells

Isolated myocytes were either cultured under normal conditions (untreated) or transfected with plasmids encoding wild type- or active-PKCδ. 48 hours post-transfection, cardiocytes were subjected to 2.5 hours simulated ischaemia and immediately harvested for Western blot analysis. Constituent proteins were probed with anti-p38 or anti-phospho-p38 antibodies to detect the effect of PKCδ overexpression on either p38 induction or activation (see Figure 4-6). Overexpression of wild type PKCδ increased p38 phosphorylation during ischaemia, although comparable results with empty vector alone suggest this is not a consequence of PKCδ (results not shown). Interestingly however, transfection of active PKCδ significantly attenuated the ischaemia-induced p38 activation, although there was no effect on total p38 levels.

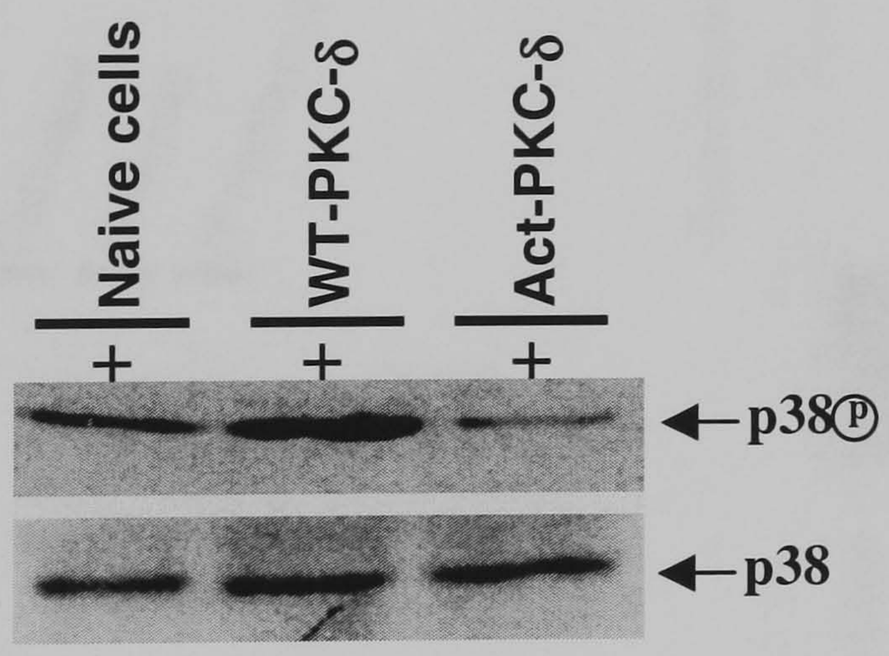

Figure 4-6: Effect of active PKCδ expression on p38 phosphorylation during ischaemia.
Untreated myocytes (Naïve cells) and cells transfected with wild type PKCδ (WT-PKCδ) or active PKCδ (Act-PKCδ) were subjected to 2.5 hours simulated ischaemia (+) and harvested for Western blot analysis to detect p38 dual phosphorylation. p38 activation was greater during ischaemia in untreated or wild type PKCδ transfected cells, compared to myocytes expressing active-PKCδ. Total p38 levels in the same cells were unchanged.

We have shown previously that cells expressing active PKCδ are protected against cell death during ischaemia (221) and, as we show here, PKCδ activation causes an inhibition of p38 phosphorylation. Since preconditioning also protects by activating PKC in this model, we sought to compare its effect on p38 activation by examining ischaemia-induced phosphorylation in naïve and preconditioned cells.

3.3.2 p38 activation during ischaemia following preconditioning

Preconditioned cardiocytes and untreated controls were subjected to 2.5 hours ischaemia to maximally activate p38. Thereafter p38 phosphorylation was assessed, as before, by immunoblotting with anti-phospho-p38 antibodies. Figure 4-7 shows that, akin to active PKCδ overexpression, preconditioning consistently inhibited p38 activation during ischaemia ($p<0.01$).

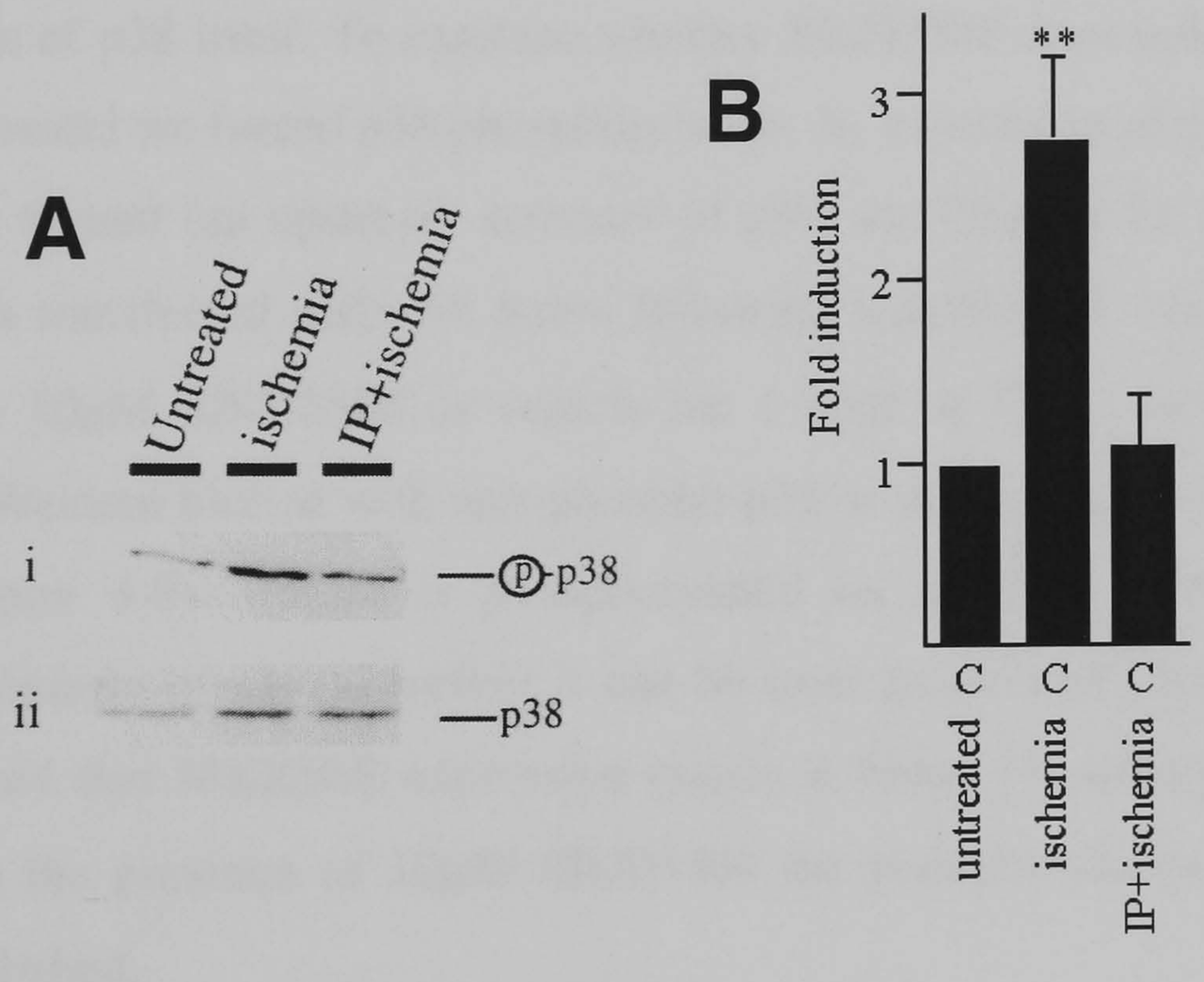

Figure 4-7: Effect of ischaemic preconditioning (IP) on the activation of p38-MAPK during ischaemia.
Panel Ai, Western blot probed with anti-phospho-p38 to detect p38 MAPK activation. Lysates were prepared from untreated cells or cells after 2.5 hours ischaemia with and without prior preconditioning. ***Panel Aii,*** total p38 levels were detected in the same cells with anti-p38 antibodies. ***Panel B,*** mean p38 activation compared to untreated controls (n=4). Simulated ischaemia causes a significant activation of p38, which is inhibited in cells preconditioned immediately prior to ischaemia. **$p<0.01$, p38 activation during ischaemia v non-ischaemic/untreated.

The negative regulation of p38 phosphorylation during ischaemia is therefore associated with the cardioprotective effects of both preconditioning and active PKCδ overexpression. Following both these cardioprotective treatments however, as well as reduced p38 activation, ischaemia and its consequences are also diminished. Therefore, on the basis of these results alone, it is not possible to ascertain whether the inhibition is a cause, or simply a result, of protection. Therefore, to define the role of p38 on cell viability following ischaemia we used pharmacological inhibition of p38 in an attempt to mimic protection afforded by preconditioning/PKC activation.

3.4 Consequence of p38 activation during ischaemia

We wished to examine the role of p38 during ischaemia by inhibiting activity with SB203580, which reversibly binds to the ATP binding site (300). SB203580 therefore prevents downstream substrate phosphorylation and does not affect the dual-phosphorylation of p38 itself. To examine whether SB203580 does indeed block p38 activity in our model we forced p38 phosphorylation, by expression of a constitutively active MKK6b mutant (an upstream activator of p38: see Chapter 5), in control and wild type p38α transfected cells. 48 hours following transfection, cardiocytes were incubated with 10μM SB203580 or vehicle for 1 hour at 37°C. Protein was then harvested and Western blotted with anti-phospho-p38 or anti-phospho (ser82)-HSP27 antibodies (Figure 4-8). HSP27 is phosphorylated on ser82 by MAPKAPK-2, a downstream substrate of p38, therefore it can be used as an assay for p38 activity. Figure 4-8 shows that MKK6bE expression causes a strong phosphorylation of p38 and HSP27. In the presence of 10μM SB203580 the phosphorylation of HSP27 is completely abolished.

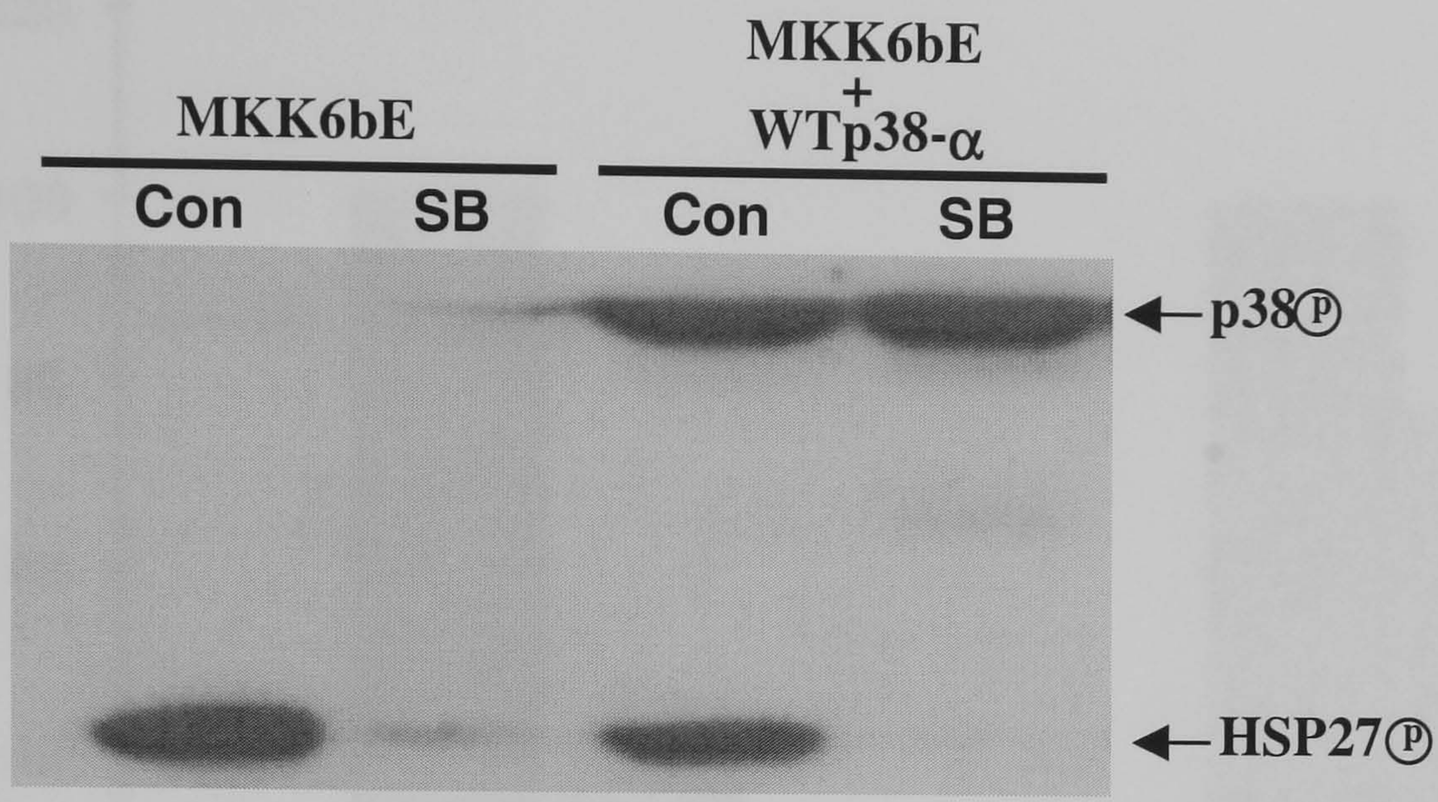

Figure 4-8: An assay for SB203580-induced p38 inhibition.
Cardiocytes, infected with MKK6bE +/- WTp38α, were treated with SB203580 (10μM) or vehicle for 1 hour. Cardiocytes were then harvested and Western blotted sequentially with anti-phospho-p38 and anti-phospho-HSP27 antibodies. Western blots demonstrate HSP27 phosphorylation only in the absence of SB203580.

Since SB203580 completely inhibits p38 activity, we next sought to examine its effect on ischaemic injury. We therefore subjected cells to 6 hours simulated ischaemia in the presence and absence of 10μM SB203580. Using two separate endpoints of creatine kinase (CK) and lactate dehydrogenase release (LDH) release to assess cell injury, and by measuring cell viability with MTT bioreduction, significant protection is observed following p38 inhibition (Figure 4-9). Therefore, on the basis of these results alone, p38 activation during ischaemia is detrimental to myocyte survival. Hence the attenuation of p38-MAPK activation by PKCδ overexpression and preconditioning at least contributes to the protective effect of both these treatments.

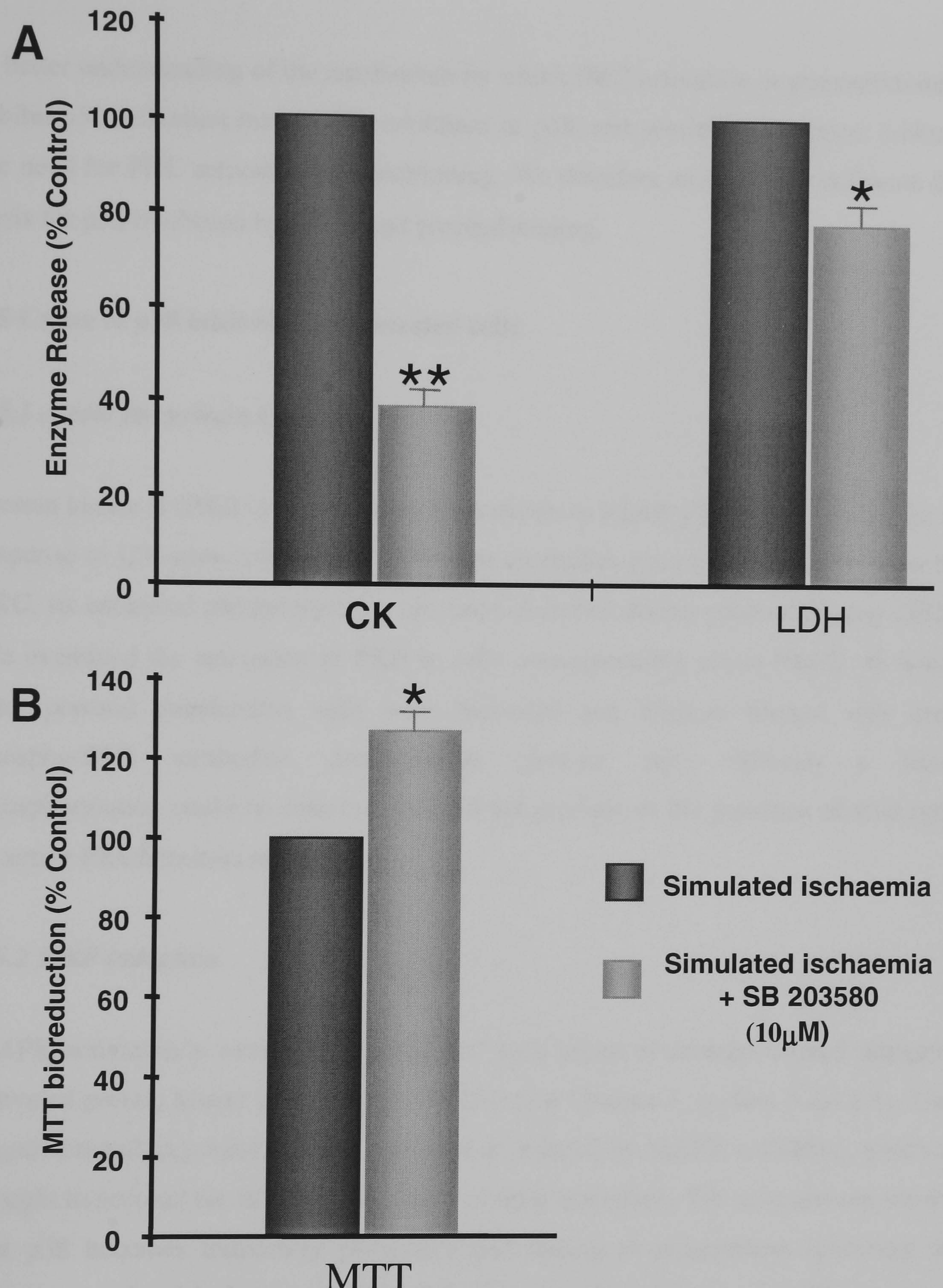

Figure 4-9: Cell viability after lethal ischaemia in the presence of SB203580.
Panel A, total CK and LDH released into ischaemia buffer during 6 hours of simulated ischaemia. Myocytes are injured less during ischaemia if p38 activation is inhibited. ***Panel B,*** cell viability was measured by MTT bioreduction within monolayers after 6 hours simulated ischaemia and 2 hours re-oxygenation. Myocytes are significantly more viable following ischaemia and re-oxygenation in the presence of SB203580. Injury in the presence of SB203580 is expressed as a percentage of injury in the absence of SB203580. Viability in cells treated with SB203580 alone for 6 hours (without ischaemia) was equivalent to that observed in untreated cells (results not shown). All *p* values are for comparisons between ischaemia in the presence and absence of SB203580. *p<0.05, **p<0.001, n=20.

A better understanding of the mechanism by which PKC activation or preconditioning inhibit p38 activation may enable inhibition of p38, and resulting protection, without the need for PKC activation/preconditioning. We therefore attempted to delineate the basis for p38 inhibition by PKCδ and preconditioning.

3.5 Cause of p38 inhibition in protected cells

3.5.1 A role for protein kinase B

Protein kinase B (PKB) has previously been shown to inhibit p38-MAPK activation in response to UV stimulation (301). Although no studies document PKB activation by PKC, its enhanced phosphorylation has been observed during preconditioning (302). We examined the activation of PKB in cells overexpressing active PKCδ. 48 hours after plasmid transfection, cells were harvested and Western blotted with anti-phospho-PKB antibodies. Immunoblots showed that, although a basal phosphorylation could be detected, this did not increase in the presence of wild type or active PKCδ (results not shown).

3.5.2 MKP induction

MAPK activation is eventually "turned off" by a group of proteins termed mitogen-activated protein kinase phosphatases (MKPs) (see Chapter 1, section 3.2.4.1.4). This negative regulatory mechanism is activated or induced by MAPK activation, which is thought to account for the transient nature of their activation. We have already shown that p38 becomes transiently phosphorylated during re-oxygenation following 90 minutes simulated ischaemia (Figure 3-9). It is possible that this MAPK activation induces/activates a MAPK phosphatase. In this sense, the phosphatase would then be capable of limiting p38 activation during subsequent ischaemia. We therefore examined the levels of MKP-1 either after active PKCδ expression or 2.5 hours following preconditioning, to determine whether activating PKCδ or preconditioning were capable of upregulating MKP protein. Again these data proved negative as no

detectable MKP-1 protein was seen in control or treated cells, although antibody affinity was confirmed with protein lysates for MKP-1 transfected cells (Dr Richard Heads, unpublished observations).

4 DISCUSSION

The studies described within this chapter investigate the relationship between preconditioning, PKC and MAPKs in primary cultures of neonatal cardiocytes. The activation of p38- and ERK1/2-MAPK during preconditioning is not downstream of PKC. Paradoxically, MAPK phosphorylation during preconditioning is inhibited by PKC activation and potentiated by PKC inhibition. A similar relationship exists during lethal ischaemia in this model following preconditioning or PKC activation. More specifically, p38-MAPK undergoes a period of prolonged phosphorylation that is inhibited by preconditioning and the expression of active PKCδ, both of which enhance myocyte survival. Inhibition of p38 activation with SB203580 also gives rise to protection. These findings suggest that p38-MAPK modulates cardiocyte survival during simulated ischaemia, which may represent a new and specific therapeutic target.

4.1 The interplay between PKC and p38 during preconditioning and ischaemia

Previous reports suggest that activation of p38 during preconditioning is responsible for the resulting protection (126, 130, 303). These conclusions were based on the ability of SB203580 to inhibit protection when given during preconditioning. Since PKC activation is also protective, we wished to examine whether this is associated with p38 activation. If p38 phosphorylation occurs downstream of PKC then we would expect overexpression of active PKCδ, which we have shown protects myocytes during ischaemia (221), to activate the p38 pathway. Our findings do not support this hypothesis, since p38 activation was inhibited, rather than activated by active PKCδ (Figure 4-2). Moreover expression of active PKCε, another PKC isoform implicated in preconditioning (107), also inhibits p38 activation during simulated preconditioning (Figure 4-3). These results were confirmed by the inhibition of PKC

with chelerythrine and staurosporine, which conversely increased p38-MAPK activation, although no effect was seen on ERK1/2 phosphorylation (Figure 4-4). These inhibitor results should be interpreted cautiously however, because staurosporine is a relatively non-specific kinase inhibitor and chelerythrine has been shown to increase p38 activation independently of PKC inhibition (304).

The inhibition of p38-MAPK by PKC has been reported in other models. In neuronal cells, PMA protects against apoptosis induced by serum withdrawal and prevents the activation of p38-MAPK. Moreover, preventing p38 activity with SB203580 also protected these cells against apoptosis (305). Conversely, in human glioma cells, inhibition of p38 with calphostin C induces apoptosis and p38 activation (306). Perhaps the greatest analogies can be made with a study by Jun and co-workers, where p38 activation and apoptosis were induced in murine macrophages by the NO donor sodium nitroprusside (SNP). In this model, the stable expression of PKCδ was sufficient to reverse both apoptosis and p38 activation (307).

Regardless of the mechanism of MAPK inhibition, these findings support a protective role for PKC activation during *lethal* simulated ischaemia, since all studies addressing the role of p38 during ischaemia, in the absence of preconditioning, demonstrate that activation is detrimental, with SB203580 decreasing infarct size and enhancing post-ischaemic functional recovery (131, 133).

4.2 MAPK activation during ischaemia in neonatal cardiac myocytes

ERK1/2 and p38-MAPK were differentially activated during ischaemia, since ERK1/2 were inhibited below basal levels throughout lethal simulated ischaemia, whereas p38-MAPK exhibited a prolonged period of phosphorylation. This is in agreement with other studies in similar models (131, 299). Also in agreement is the protection that accompanies p38 inhibition with SB203580. These data suggest that p38 activation at least contributes to injury during ischaemia. The mechanism by which p38 promotes injury is unknown, although its inhibition has been shown to

attenuate apoptosis (132) and tumour necrosis factor alpha (TNFα) (308) release from cardiomyocytes.

4.2.1 Induction of apoptosis

Programmed cell death, or apoptosis, differs from necrosis in that it does not evoke an inflammatory response due to the maintenance of sarcolemmal integrity. Instead, the cells are removed by phagocytosis from either neighbouring cells or macrophages. Following myocardial infarction in humans a significant number of cells undergoing apoptosis were detected using immunohistochemical staining to define in detail the histology and cytology of the apoptotic areas (177). In fact, the large number of apoptotic bodies lying free in the extracellular space suggested that the phagocytic capacity was overwhelmed, which may result in a deviation from apoptosis to necrosis. If this is the case, it is difficult to accurately assess the extent of apoptosis because cells that appear necrotic may simply represent a late stage of apoptosis. Nevertheless, it is likely that apoptosis at least contributes to injury following and/or during ischaemia in cardiac myocytes (see Chapter 1, section 3.3.2).

p38-MAPK activation has been shown to be promote apoptosis in a number of cell types (117, 309-311), including cardiac myocytes (312). p38 activation has been linked to apoptosis through a number of signalling pathways, which will be discussed in detail below.

4.2.1.1 p53

p38 causes the phosphorylation of the pro-apoptotic gene p53 on serine 389 (313, 314) and serine 33 (315), which stimulates the functional activity of this enzyme. In neonatal rat cardiac myocytes, p53 is activated by hypoxia, and artificially increasing activity by p53 overexpression promotes cardiomyocyte, but not fibroblast, apoptosis (316). Furthermore, in the isolated rat heart p53 protein is upregulated during reperfusion following regional ischaemia (317), although it should be noted that apoptosis following hypoxia or global ischaemia is unaltered in myocytes, and

Langendorff-perfused hearts, from p53 knockout mice (318). Therefore, there is no doubt that p53 activation promotes apoptosis, and it is possible that p53 activation occurs in the heart during ischaemia/reperfusion injury. The effect of ischaemic preconditioning on p53 activation has not been addressed in the heart, but the activation of p53 and its target genes during forebrain ischaemia in the rat is markedly inhibited by prior ischaemic preconditioning (319). This parallels the activation of p38 in our model, which is activated during ischaemia but inhibited by prior preconditioning. Therefore the role of p53 during ischaemia/reperfusion in the heart is controversial, however the possibility that p38 promotes injury through p53 activation warrants investigation.

4.2.1.2 Signal transducers and activators of transcription (STATs)

p38-MAPK activity is required for the serine phosphorylation of signal transducer and activator of transcription-1 (STAT-1) in response to a variety of cytokines including interferon-α, β, and γ (IFN-α/β/γ) (320, 321), and interleukin-2 and 12 (IL-2/12) (322). This serine phosphorylation leads to STAT-1 activation and apoptosis in a variety of models (320, 323), including rat neonatal cardiac myocytes (324). Moreover, this has physiological relevance to ischaemia, because overexpression of STAT-1 or depletion of STAT-1 with antisense oligonucleotides augments or attenuates ischaemia-induced cell death respectively (324). Although this study uses a similar model of prolonged simulated ischaemia in neonatal cardiac myocytes, the exact time of STAT-1 phosphorylation was not addressed. Therefore, it would be interesting to assess STAT-1 phosphorylation throughout prolonged ischaemia to ascertain whether it shadows p38 phosphorylation, and more importantly, whether it is inhibited by SB203580.

4.2.1.3 Cytochrome C

Ultraviolet B (UVB) radiation causes sustained activation of p38 and apoptosis in HaCaT cells (321). In this model, p38 promotes apoptosis by mediating the release of cytochrome C into the cytosol, because inhibition of p38 prevented apoptosis and

cytochrome C release from mitochondria. During ischaemia/hypoxia in adult ventricular myocytes, cytochrome C release has been detected in combination with apoptosis (325). Moreover, inhibition of cytochrome C release is associated with the anti-apoptotic action of ARC (an anti-apoptotic protein that is expressed predominantly in heart and skeletal muscle) in a heart derived cell line (H9c2), implying that cytochrome C release maybe a cause rather than a consequence of apoptosis (326). Again, the role of p38 in mediating the release of cytochrome C during ischaemia should be addressed. Interestingly, the fall in ATP levels during hypoxia has been linked to the translocation of Bax to the mitochondria and the subsequent release of cytochrome C (327). This is a potential mechanism for p38-mediated cytochrome C release, since p38 promotes Bax translocation to the mitochondria during nitric oxide-mediated apoptosis in neurons (328).

4.2.2 Tumour necrosis factor

Tumour necrosis factor alpha (TNFα) is released from the heart following ischaemia in animal models (329), and following cardiopulmonary bypass surgery in humans (330). Moreover, circulating levels of TNFα are elevated after myocardial infarction, which correlates with the level of myocardial injury (331). The consequences of TNFα on the heart include apoptosis, necrosis and contractile depression (332, 333). Thus, the release of TNFα during ischaemia and the corresponding detrimental effects on cardiac myocytes, suggests it at least contributes to myocardial injury following ischaemia and/or reperfusion.

TNFα is regulated at multiple levels by p38-MAPK. Firstly, the release of TNFα is regulated at the transcriptional and translational level by p38 (334, 335). Secondly, some cellular responses to TNFα treatment are also dependent upon p38 activation (336, 337). Thus, p38-MAPK is associated with both the release and action of TNFα. The release of TNFα in a human model of myocardial ischaemia in isolated trabeculae is dependent on p38 activity (135). Moreover, inhibition of p38 not only decreases TNFα release but also enhances post-ischaemic myocardial function.

Furthermore, the protection against oxidant stress in isolated perfused rat hearts seen following p38 inhibition is also associated with a decrease in TNFα production (308). Thus, the activation of p38 during prolonged simulated ischaemia in our model may cause injury by promoting the release of TNFα from myocytes. If this is true, then the decreased TNFα release during reperfusion in preconditioned rat hearts (338) maybe a result of decreased p38 activation during ischaemia.

4.3 A role for other MAPKs in cell death?

During ischaemia as well as p38 activation, we observe a strong suppression of ERK1/2-MAPK phosphorylation. It has been reported that a decrease in ERK1/2 phosphorylation may be as important as increased p38 activation in triggering cell death (115). An observation supported by the fact that pharmacological inhibition of ERK1/2 with PD98059 causes apoptosis through activation of p38 in HeLa cells (301). In addition, ERK1/2 phosphorylation is reported to be both necessary (339) and sufficient (223) for protection in the whole heart. In our model however, preconditioning does not increase ERK1/2 activation during ischaemia (results not shown), which reinforces the importance of understanding cell death pathways independently of preconditioning since the manipulation of ERK1/2, in addition to p38, may further increase protection. Conversely preconditioning may activate deleterious pathways, the inhibition of which could potentiate protection.

4.4 The effect of preconditioning on p38 signalling during ischaemia

Regardless of the mechanism of p38-induced injury, it is clear that inhibiting its activity during ischaemia protects. It is also known that PKC activation prevents p38 phosphorylation in this model, which may be the mechanism by which PKC activation protects against lethal ischaemia. Indeed we did observe a decreased activation of p38 during lethal ischaemia in cells expressing constitutively active PKCδ. Preconditioning is also known to depend on the activation of PKC, therefore it may protect by similarly inhibiting p38 activation during ischaemia. As shown in Figure 4-7, preconditioning significantly inhibited p38 activation during ischaemia.

These data provide the first mechanistic link between ischaemic preconditioning and reduced p38 activation during ischaemia. Insights into the mechanism by which PKC/preconditioning inhibit p38 may reveal new therapeutic pathways.

4.5 The mechanism of reduced p38 activation

4.5.1 Mitogen-activated protein kinase phosphatases

MAPK pathways relay, amplify and integrate signals from a diverse range of stimuli to elicit an appropriate physiological response. One critical determinant of physiological outcome is the duration and magnitude of MAPK activation, which may be regulated at multiple points within the MAPK pathway. Ultimately, the activity of MAPK reflects a balance between the activities of the upstream kinase and the protein phosphatases. Therefore, the phosphatases play an equally important, often overlooked, role in MAPK regulation. The best-characterised family of proteins capable of dephosphorylating MAPKs are the mitogen-activated protein kinase phosphatases (MKPs: Chapter 1, section 3.2.4.1.4). Many of the stimuli that activate MAPK, similarly increase MKP activity, which then act as a negative feedback loop by dephosphorylating MAPK and turning off the signal (340).

MKP-1 induction can be stimulated by PKC activation (341). Therefore, perhaps MKP-1 induction is responsible for the suppressed MAPK activation in active PKCδ/ε expressing cells. Unfortunately, the levels of MKP-1 protein were undetectable 48 hours following active PKCδ transfection. MKP-1 expression is also hypoxia responsive, since it is induced by low oxygen conditions (342). Therefore, the sublethal ischaemia of preconditioning may upregulate MKP-1 protein. Furthermore, stimuli that activate MAPK are also thought to activate MKPs, and we have already shown that MAPKs are activated during preconditioning in this model. Using antibodies raised against MKP-1 protein, we observed no detectable increase in MKP-1 protein 3 hours following ischaemic preconditioning (at the time-point when p38 phosphorylation is attenuated). This does not discount the involvement of another phosphatase however. In fact, inhibition of tyrosine phosphatases with vanadate

prolongs p38 activation and increases susceptibility to simulated ischaemia in neonatal myocytes (134).

If a phosphatase, capable of dephosphorylating MAPKs during ischaemia, is induced/activated following preconditioning, it maybe a consequence of MAPK activation during preconditioning. In this scenario, the protection would be dependent upon MAPK activation during preconditioning and MAPK inhibition during lethal ischaemia. For example, inhibition of MAPK during preconditioning would prevent phosphatase induction and therefore attenuate protection, whereas inhibition of p38 during ischaemia would prevent injury and thus protect. Many reports in the literature suggest this exact dichotomy. Inhibition of p38 during preconditioning aborts protection (126, 130, 303), whereas p38 inhibitors during ischaemia protect (131, 133). In fact the only study to address the role of p38 during both preconditioning and ischaemia found a similar dichotomy, with SB203580 preventing protection when given during preconditioning, but protecting when applied during ischaemia alone (299).

Although MKP-1 induction was not observed following ischaemia during hypoxic preconditioning in our model, induction is observed following preconditioning in a similar model within our laboratory, which relies on metabolic inhibition with 2-deoxyglucose and dithionite, instead of hypoxia, to precondition. In this model a one-hour metabolic stress preconditions and causes a similar activation of p38 during re-oxygenation. p38 soon becomes dephosphorylated below basal levels within 1 hour of activation during re-oxygenation. This dephosphorylation is concomitant with the expression of MKP-1 protein. MKP-1 levels remain elevated for a further 6 hours, during which time p38 levels remain depressed. Moreover, preliminary experiments suggest that transfection of MKP-1 in this model inhibits p38 activation, although it's corresponding effect on cell viability remains to be determined (Dr Richard Heads, unpublished observations).

4.5.2 Protein kinase B

It is not only phosphatases that can inhibit MAPK activity, because kinases can also prevent p38 activation. In HeLa cells the inhibition of ERK is sufficient to stimulate p38 activation and apoptosis, although addition of serum is sufficient to abolish this increased activity (301). In this model, inhibition of phosphatidylinositol 3-kinase (PI-3 kinase) with wortmanin, or PKB inhibition with a dominant negative mutant, prevents the reversal of apoptosis/p38 activation with serum. This suggests that the PI-3 kinase/PKB pathway is able to negatively regulate p38. PKB activation does not appear to contribute to p38 inhibition in response to active PKCδ expression because no detectable PKB phosphorylation (Ser473 and Tyr308) was observed in active PKCδ expressing cells. The inhibition of p38 in response to simulated preconditioning maybe related to PKB, since PKB activation has been reported during preconditioning in this model (302). Unfortunately, studies in our laboratory suggest this is not the case since p38 activation during preconditioning is not modulated by PI-3 kinase inhibition with wortmanin (Dr James Mockridge, unpublished observations).

4.5.3 14-3-3

14-3-3 family members are dimeric phospho-serine binding proteins that participate in signal transduction pathways. In cultured fibroblasts, expression of dominant negative 14-3-3-zeta increases the basal activation of p38 and JNK as well as promoting apoptosis (343). This induction of apoptosis was prevented by the inhibition of p38. More interestingly, targeted expression of dominant negative 14-3-3-zeta to murine postnatal cardiac tissue increased basal p38 activity and caused widespread apoptosis. The activation of 14-3-3 has not been assessed in relation to ischaemia, but is role as an anti-apoptotic signalling protein in the heart, coupled with its ability to inhibit p38, makes it a candidate binding protein for preconditioning in this model.

4.5.4 Differential p38 localisation

For p38 to be phosphorylated it must be in close proximity to its upstream activator. During ischaemia, p38 may localise to active upstream kinase or the co-localised kinase may simply be phosphorylated. In either instance, changing p38 localisation may alter p38 phosphorylation. Therefore preconditioning may simply relocate p38 away from its upstream activator, therefore preventing phosphorylation during subsequent ischaemia. Conversely, preconditioning may prevent the relocation of p38 during ischaemia, thus inhibiting phosphorylation. To address these hypotheses, studies will be needed to examine the mechanism of p38 activation during ischaemia. This can then be compared to cells made ischaemic following preconditioning.

Although the mechanisms listed above suggests that preconditioning may inhibit p38 phosphorylation directly, it may simply inhibit/downregulate a kinase upstream of p38. To examine this possibility one can assess the activation of upstream kinases (MKK3/6) during ischaemia in control and preconditioned cells.

4.6 Critique of methods

The activation of p38 during simulated ischaemia can be quantified not simply by the level of phosphorylation (which is maximal after 2.5 hours ischaemia) but also by the duration. Therefore the duration of p38 activation is a critical determinant of cell outcome following ischaemia. Treatments that prolong p38 activation in similar models increase cell injury (134). Therefore, when assessing the modulation of p38 activation by preconditioning/PKC activation, one should address the effect on the temporal pattern of p38 activation. It may be that a treatment decreases the maximal activation, but prolongs the total activation. Furthermore, preconditioning may simply delay the activation of p38 during ischaemia, thereby reducing the activation at 2.5 hours, but having no effect on total activity. The abolition of p38 phosphorylation after 2.5 hours ischaemia suggests however, that total activation is at least decreased. Nevertheless, in the absence of time constraints, the time course of p38

phosphorylation during ischaemia should be quantified in control and preconditioned cells

Increasing numbers of studies have questioned the specificity of the p38 inhibitor SB203580 used in these experiments. As well as inhibition of other MAPKs (344), SB203580 is also known to inhibit PDK1 (345) and activate the serine/threonine kinase raf-1 (346). The concentrations required to elicit these effects however, are thought to be greater than 1μM and parallel experiments within our laboratory have demonstrated protection against simulated ischaemia using 1μM SB203580 (Dr Roby Rakhit, unpublished observations).

4.7 Conclusions

Selective activation of PKC isoforms or ischaemic preconditioning attenuates the phosphorylation of p38 during ischaemia and protects. Ischaemic preconditioning requires the activation of PKC for protection, thus we hypothesise that PKC activation during preconditioning is also required for the reduction in p38 activity. It would be interesting to determine whether known inhibitors of preconditioning (i.e. GPCR antagonists and PKC inhibitors) prevent the reduction in p38 activation during ischaemia.

We also know that p38 inhibition is protective in this model, therefore the reduction in ischaemia-induced p38 activation following preconditioning at least contributes to the resulting protection. The relative contribution of p38 inhibition to protection is unknown, although the levels of injury seen following p38 inhibition in these studies, and preconditioning in others studies within our group (221), are similar.

The mechanism by which p38 phosphorylation is attenuated during ischaemia following preconditioning in this model remains unknown. The reduction in p38 activation may be a consequence of attenuated activation or enhanced dephosphorylation. However, if the latter is the case then preconditioning should

diminish the activation of p38 following other known stimuli in the absence of ischaemia.

Chapter 5. p38 isoform activation during ischaemia

1 INTRODUCTION

Like many other protein kinases, p38-MAPK comprises a family of structurally related proteins. To date, five separate mammalian p38 isoforms have been identified: p38α (347, 348), p38β1/β2 (349, 350), p38γ (351, 352), and p38δ (353, 354). Recent reports suggest that these isoforms may differ significantly in function if not in structure. For example, the specific inhibition of p38α and β in HeLa cells, by the pyridinyl imidazole SB202190, induces apoptosis (355). Expression of p38β attenuates this effect, whereas p38α expression promotes cell death. A similar 'yin-yang' role for p38α and β has been demonstrated by Wang and coworkers in neonatal cardiac myocytes. In this model, overexpression of a constitutively active mutant of the upstream p38 activator MKK3, in combination with p38α, promoted apoptosis (116). Conversely, overexpression of constitutively active MKK6 in combination with p38β promoted hypertrophy. Moreover, these effects could be negated by the expression of dominant negative p38α or β respectively.

The dual-phosphorylation specific antibodies used to assay p38 activity recognize the TGY motif, and surrounding amino acids, present in p38 isoforms. Unfortunately, the sequence surrounding the TGY motif is conserved in all p38 isoforms, thus phosphorylation-specific isoform-selective antibodies are unavailable. Therefore, the measurement of total p38 activation, using phospho-specific antibodies has limitations. For example, an increase in p38α activation with a comparable decrease in p38β phosphorylation may not alter total p38 activation as detected by phospho-specific antibodies. If the Wang hypothesis were correct however, we would expect such a change in the balance of active p38 isoforms to cause a large decrease in cell viability. Thus there is a possibility that this decrease in viability may not be correctly attributed to p38 activation using currently available antibodies. Furthermore, the differing effects of p38 activation on myocyte survival, such as the protection and injury caused by p38 phosphorylation during preconditioning (130) and ischaemia (131), may stem from the activation of different p38 isoforms. Therefore we wished to examine the isotype specific activation of p38 during ischaemia in our model.

2 SPECIFIC METHODS

2.1 Experimental protocols

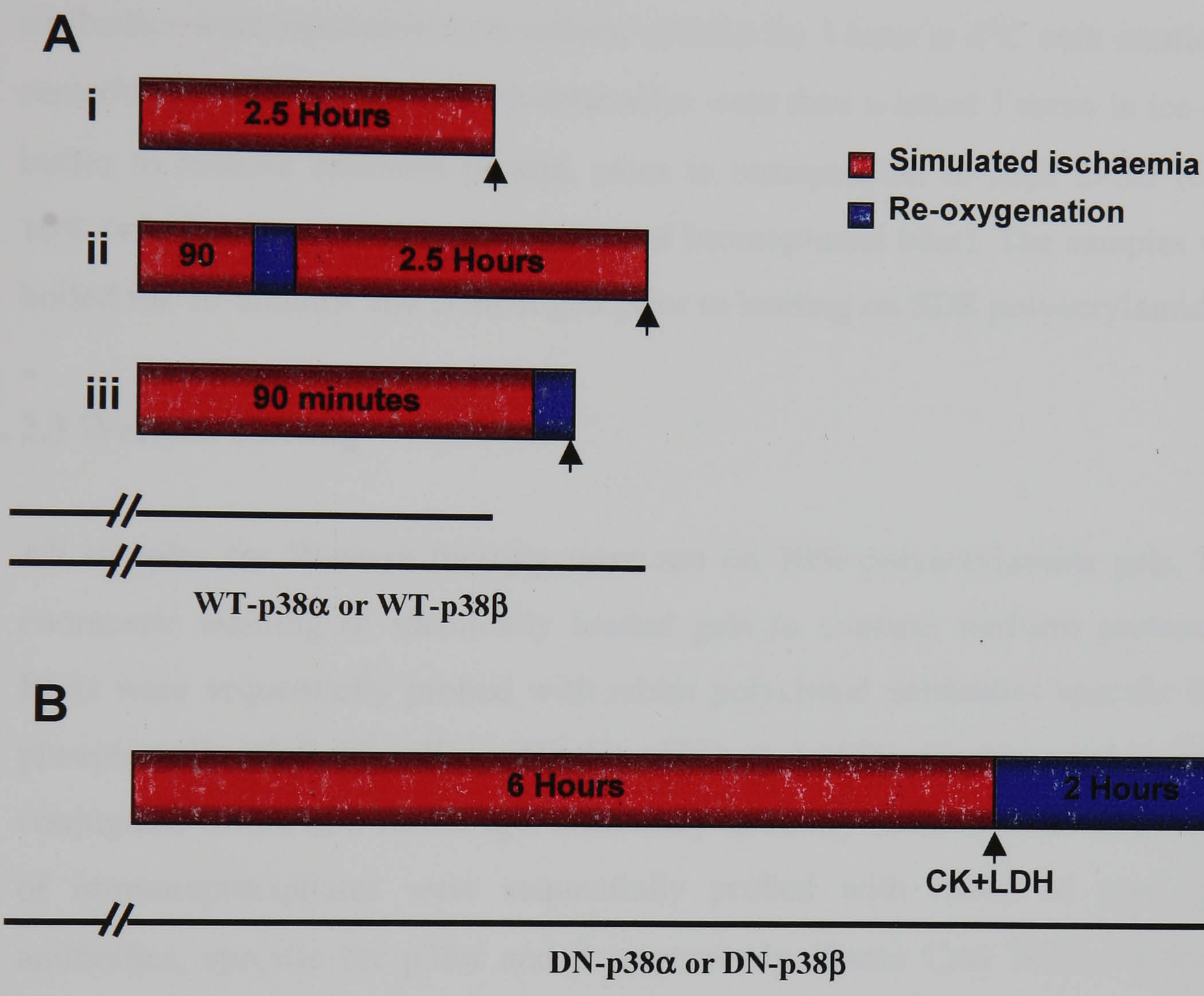

Figure 5-1: Schematical representation of experimental protocols used to assess the consequence and extent of p38 isoform activation during ischaemia.
Panel Ai and ii, myocytes expressing wild type p38α or p38β were preconditioned (ii) or left untreated (i) before exposure to simulated ischaemia for 2.5 hours. ***Panel Aiii,*** p38 isoform activation during preconditioning was assessed by subjecting wild type-p38α or β-transfected cells to 90 minutes simulated ischaemia and 10 minutes re-oxygenation. Protein was harvested, as indicated by the arrows, for Western blot analysis with anti-p38 antibodies. ***Panel B,*** 24 hours after infection with dominant negative p38α or p38β, cells were subjected to a 6-hour lethal ischaemic stress. At the end of ischaemia enzyme release was determined, whereas cell viability was determined after a further 2 hours re-oxygenation.

2.2 Immunoprecipitation of p38 isoforms

At indicated time-points, myocytes were washed briefly in ice-cold PBS. The PBS was aspirated and 100µl cell fractionation buffer containing 1% (v/v) triton X-100

added to each well. The cell suspension from each well was scraped into a microcentrifuge tube and incubated with shaking at 4°C for 20 minutes, prior to centrifugation to remove insoluble material. The supernatant was centrifuged again in new tubes and added to fresh tubes containing 2.5µl of immobilised-phospho-p38 mouse monoclonal antibodies (Cell Signalling, Hitchin, UK). The immobilised-antibodies were incubated with cellular protein for 1 hour at 4°C with rotation, before centrifugation. The immobilised-antibodies were then washed 3 times in ice-cold lysis buffer to remove unbound protein, prior to resuspension in 50µl 2×SB (containing 10% (v/v) 2-mercaptoethanol and trace of bromophenol blue). The samples were then boiled for 10 minutes and centrifuged prior to loading on SDS polyacrylamide gels.

2.3 Western blotting

All samples for Western blotting were run on 10% polyacrylamide gels, following coomassie staining of identically loaded gels to confirm uniform protein loading. Blots were sequentially probed with rabbit polyclonal antibodies specific for p38 or phospho-p38 (Cell Signalling, Hitchin, UK) at 1:1000 dilutions and a peroxidase-conjugated swine anti-rabbit IgG secondary antibody (DAKO A/S, Denmark). Blots of immunoprecipitates were sequentially probed with rabbit or goat polyclonal antibodies, specific for p38α and β respectively (Santa Cruz Biotechnology, Santa Cruz, USA), and peroxidase-conjugated swine anti-rabbit or rabbit anti-goat IgG secondary antibodies (DAKO A/S, Denmark). Primary antibodies were incubated at either 1:500 (p38α) or 1:5000 (p38β) overnight at 4°C, prior to incubation with secondary antibodies at a 1:2500 dilution for 2 hours at room temperature. For translocation experiments, mouse monoclonal antibodies for phospho-MKK3/6 (Santa Cruz Biotechnology, Santa Cruz, USA) were used at 1:1000 dilutions overnight prior to a 2-hour exposure to relevant secondary antibodies as above.

2.4 Statistical analysis

All values are expressed as mean±SEM. Data for individual treatments were collected from no more than two wells from each experimental preparation. The "n" numbers

under "results" relate to the number of wells from which data were obtained. For each treatment mean values were pooled to allow statistical comparisons. Statistical comparisons between groups were performed by one-way analysis of variance (ANOVA), followed where appropriate by the Tukey-Kramer test for pair-wise comparisons. All analyses were performed using Statview version 4.0 statistical package (Abacus Concepts Inc., Berkeley, CA). A probability value ≤ 0.05 was considered significant.

3 RESULTS

3.1 p38 isoform activation during ischaemia

3.1.1 Activation of ectopically expressed p38α and β

As discussed earlier, phosphorylation-specific p38 antibodies do not selectively detect individual p38 isoforms. To look at the activation of individual isoforms, we utilised recombinant adenoviruses as an efficient gene delivery vector to express various p38 signalling molecules (116, 356). Using a recombinant adenovirus expressing the green fluorescent protein (GFP) as a reporter, greater than 95% of myocytes express the transgene 48 hours post-transfection (results not shown). Cardiomyocytes were infected with vectors expressing FLAG-tagged wild type-p38α and -p38β. Adenovirally-encoded p38β has a higher apparent Mw than p38α, thus enabling us to easily distinguish between the isoforms using p38 antibodies (Figure 5-2, *Panel A*). At a multiplicity of infection of 10, adenoviral-directed p38α and p38β expression was detected at comparable levels by Western blot analysis (Figure 5-2, *Panel Aii*).

Using phospho-specific antibodies to examine activation, we noted that during ischaemia p38α exhibits a strong phosphorylation (Figure 5-2, *Panel Ai*). In contrast transfected p38β, which exhibits a high level of basal phosphorylation, is inhibited during ischaemia (Figure 5-2, *Panel Ai*). This experiment was repeated at least four times, and band densities analysed with respect to basal phosphorylation of each

isoform in the control untreated cells. Thus activation (phosphorylation) during ischaemia is expressed as fold-increase over baseline. The results show that ectopically expressed p38α and β are differentially regulated during ischaemia, with p38α being activated whereas p38β is inhibited (Figure 5-2, *Panel B*; p<0.001).

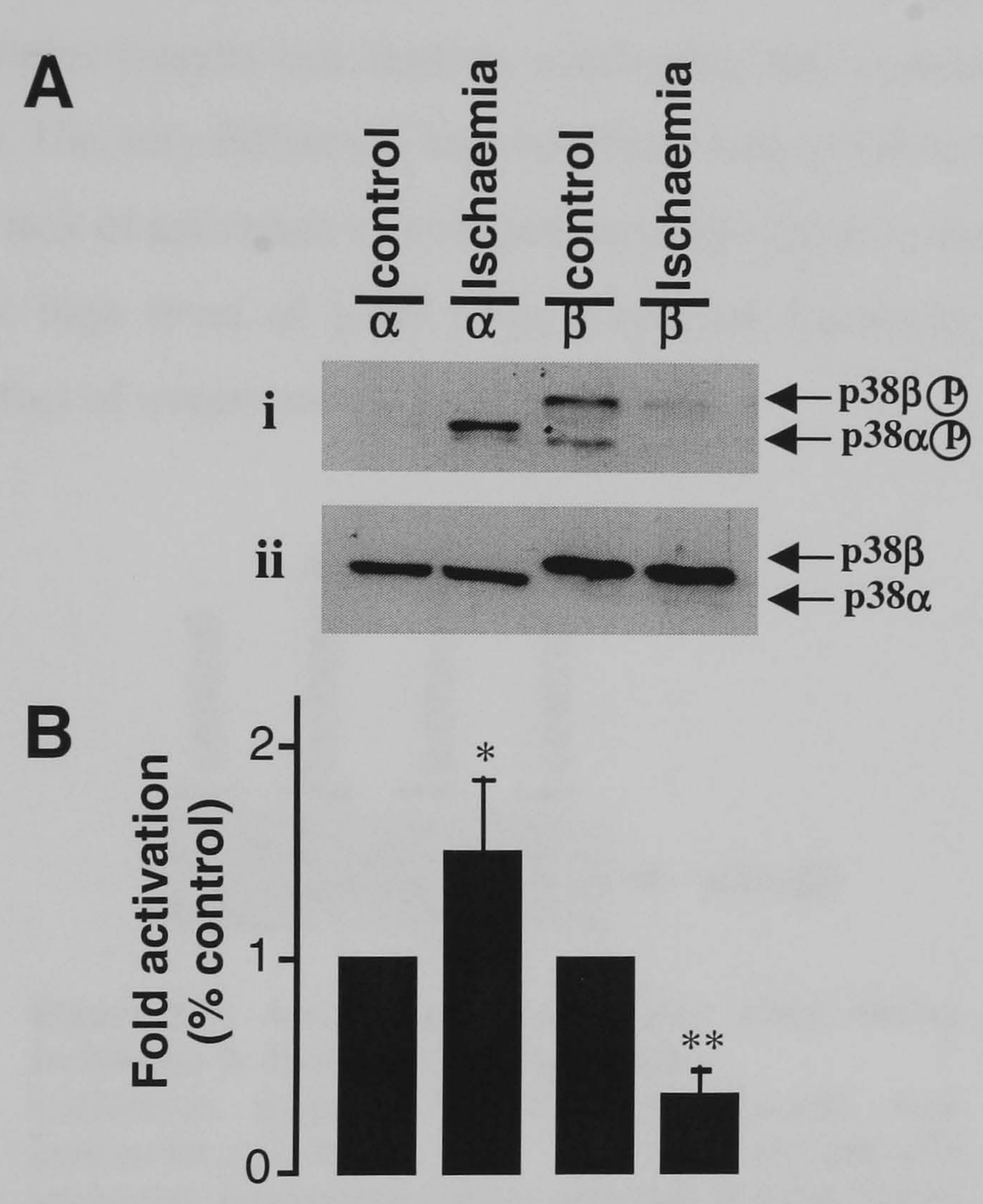

Figure 5-2: Activation of ectopically expressed p38α and p38β during ischaemia in neonatal cardiac myocytes.
Cardiocytes were infected with adenoviral constructs encoding FLAG-tagged wild type p38α or β. 48 hours post-infection, cardiocytes were subjected to 2.5 hours simulated ischaemia before cells were harvested and lysed for Western blots. ***Panel A,*** lysates were probed with dual-phospho-specific p38 antibodies to detect isotype-specific p38 activation during ischaemia (i). The expression levels of p38α and β were similar and remain unaltered between treatments (ii). ***Panel B,*** mean data shows that p38α is activated during ischaemia, but p38β is significantly inhibited. *p<0.001, p38α activation v p38β activation during ischaemia. **p<0.01, p38β activation during ischaemia v non-ischaemic/untreated cells.

3.1.2 Activation of endogenous p38α and β

Ischaemic phosphorylation of overexpressed p38 isoforms does not necessarily mimic the physiological activation of p38 isoforms. Therefore endogenous p38 isoform activation was assessed, by immunoprecipitation of phosphorylated p38 during

ischaemia, and Western blotting of the immunoprecipitates with the respective isoform-specific p38 antibodies. Cardiocytes were left untreated (control) or subjected to 2.5 hours simulated ischaemia (ischaemia). p38α could only be detected in immunoprecipitates from ischaemic cells, indicating activation of this endogenous isoform (Figure 5-3). p38β activation however, could not be detected in either control or ischaemic samples (results not shown), confirming this isoform is not activated during ischaemia. The only difference between these data and those using ectopic p38 expression is the lack of activation of endogenous p38β under control conditions. This suggests that the high level of p38β phosphorylation following transfection may simply be an artefact of overexpression.

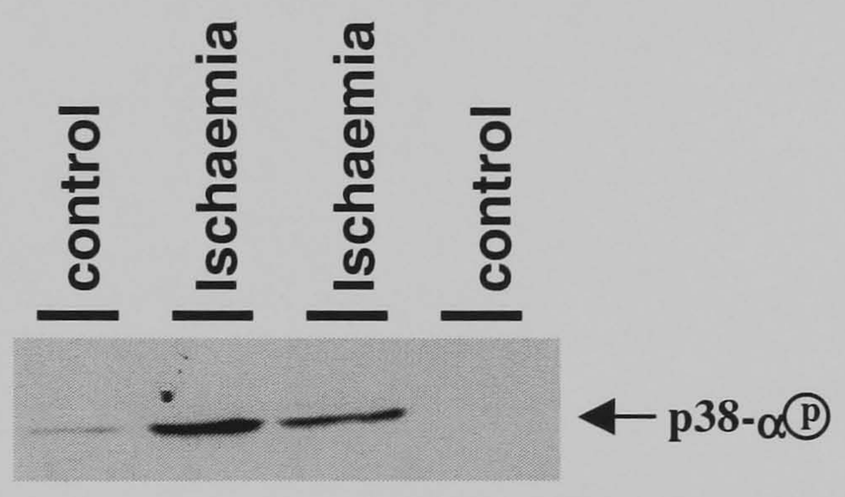

Figure 5-3: Activation of endogenous p38α during Ischaemia in neonatal cardiac myocytes.
Cardiocytes subjected to 2.5 hours ischaemia were immunoprecipitated with anti-dual-phospho-p38 antibodies. Immunoprecipitates were then Western blotted with p38α antibodies. There is a clear increase in dual-phospho-p38α during ischaemia in both independent experiments.

3.2 p38 isoform activation during ischaemia following preconditioning

On the basis of the results above we can conclude that the endogenous p38 isoform activated during ischaemia is p38α. We also know, from the data presented in the previous chapter, that p38 activation during ischaemia is attenuated in preconditioned cells. Therefore, we wished to examine the effect of preconditioning on ectopically expressed p38α phosphorylation during ischaemia. 48 hours following adenoviral-infection of p38α or p38β, expression levels are similar in all treatment groups (Figure 5-4, *Panel Aii*). Although p38α is again phosphorylated during ischaemia, in

preconditioned cells this phosphorylation is markedly diminished (Figure 5-4, *Panel Ai*). Examining the mean data, we see the results for p38α mirror those of endogenous p38, in that it is activated during ischaemia in control, but not preconditioned, cells ($p<0.05$). In contrast, transfected p38β exhibits a high level of basal phosphorylation and is inhibited during ischaemia ($p<0.01$) with no significant effect of preconditioning on this ischaemia-induced inhibition (Figure 5-4, *Panel B*). Thus preconditioning selectively inhibits the activation of p38α.

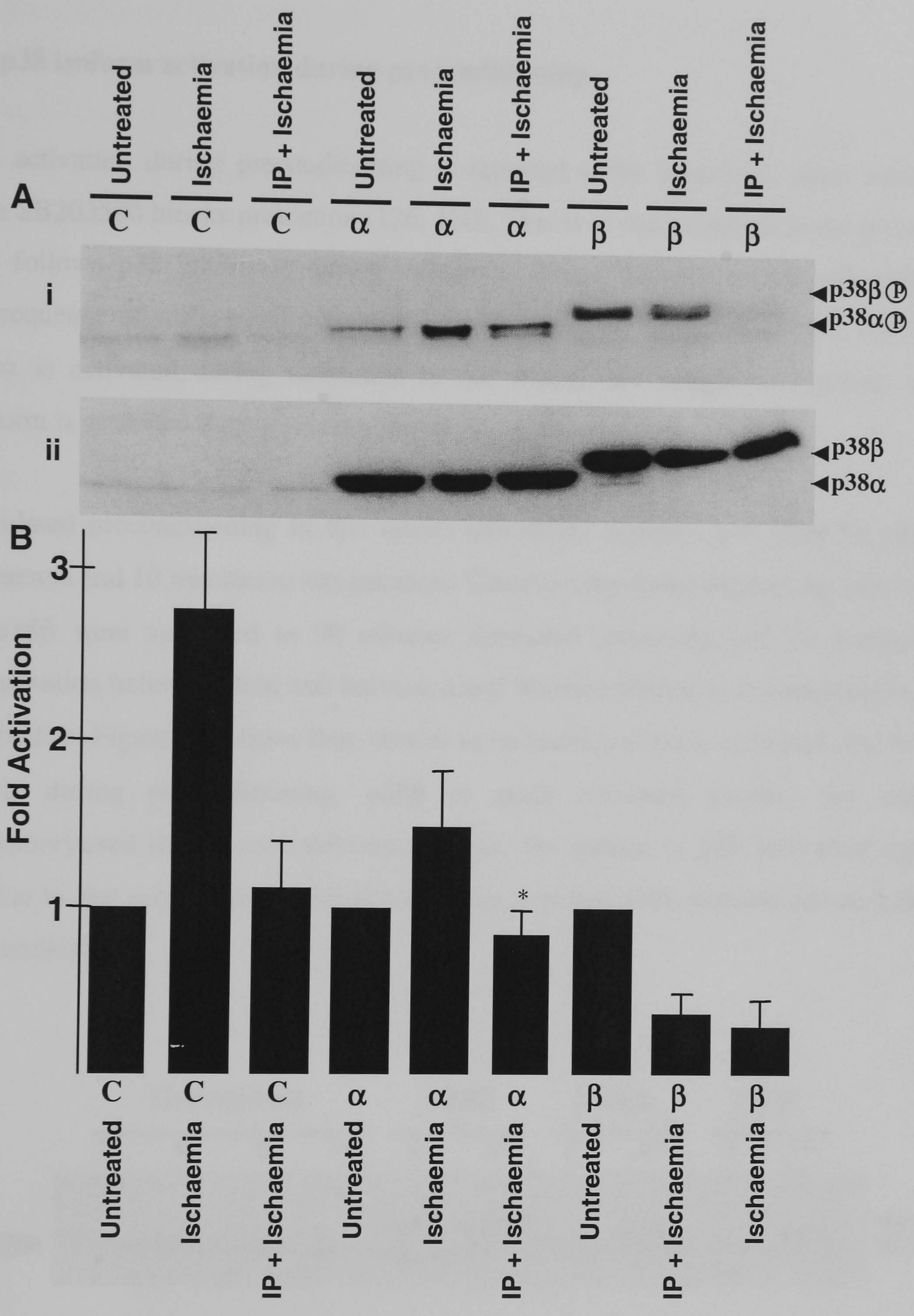

Figure 5-4: Activation of p38 isotypes during ischaemia in untreated and preconditioned cells. Cardiocytes were infected with adenoviral constructs encoding FLAG-tagged wild type p38α or β. 48 hours post-infection, untreated and preconditioned cardiocytes were subjected to 2.5 hours simulated ischaemia before cells were harvested and lysed for Western blots. ***Panel A,*** lysates were probed with dual-phospho-specific p38 antibodies to detect isotype-specific p38 activation during ischaemia (i). The expression levels of p38α and β were similar and remain unaltered between treatments (ii). ***Panel B,*** mean p38 activation during ischaemia compared to untreated controls in at least 4 independent experiments. The activation of p38α during ischaemia is inhibited in preconditioned cells, similar to the endogenous p38 in untreated controls. *$p<0.05$, p38α activation during ischaemia with v without preconditioning (IP).

3.3 p38 isoform activation during preconditioning

p38 activation during preconditioning is reported to be beneficial, since inhibition with SB203580 blocks protection (126, 130). This is in stark contrast to the protection that follows p38 inhibition during ischaemia. These dichotomous results maybe a consequence of differential activation of p38 isoforms. Since we already know that p38α is activated during ischaemia in our model, we sought to examine which isoform is activated during preconditioning.

Simulated preconditioning in this model maximally activates p38 after 90 minutes ischaemia and 10 minutes re-oxygenation. Therefore myocytes expressing either p38α or p38β were subjected to 90 minutes simulated ischaemia and 10 minutes re-oxygenation before protein was harvested and Western blotted with anti-phospho-p38 antibodies. Figure 5-5 shows that, similar to ischaemia, p38α is activated above basal levels during preconditioning. p38β is again activated basally, but remains phosphorylated during preconditioning. Thus, the pattern of p38 activation appears similar to that seen during lethal ischaemia, except that p38β remains elevated during preconditioning.

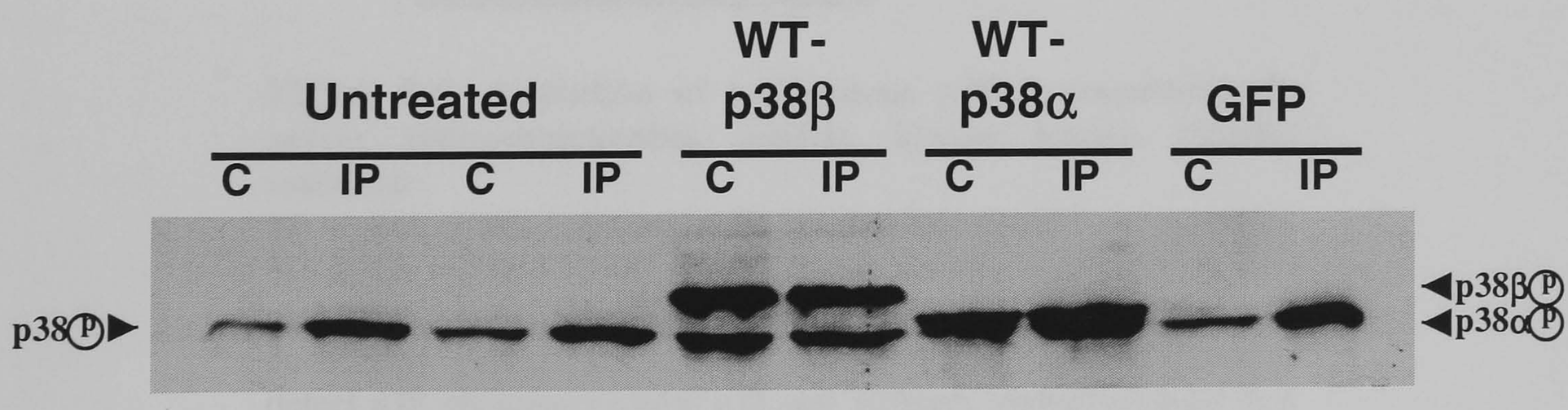

Figure 5-5: Activation of ectopically expressed p38 isoforms during preconditioning.
Cardiocytes were infected with adenoviral constructs encoding GFP, FLAG-tagged wild type p38α or β. 48 hours post-infection, cells were harvested (C) or subjected to 90 minutes simulated ischaemia and 10 minutes re-oxygenation (IP), before harvesting and Western blotting with anti-dual-phospho-p38 antibodies. The phosphorylation of p38α is elevated during preconditioning, whereas p38β remains activated.

3.4 The cause of differential p38 isoform activation during ischaemia

It has been suggested that p38 isoforms are differentially activated by their upstream activators, MKK3 and MKK6, with MKK3 preferentially activating p38α (357, 358). If this selectivity is present in neonatal cardiac myocytes then ischaemia may activate only MKK3, which would result in selective p38α phosphorylation. Therefore we wished to test for selectivity in myocytes, by transfecting with constitutively active MKK3bE and MKK6bE mutants (mutants of MKK3 and MKK6, which mimic an activating dual-phosphorylation; described previously (359)) in combination with p38α or β. Firstly, the MKK3bE and MKK6bE were transfected alone into myocytes, which were harvested 48 hour later for Western blotting with anti-phospho-p38 antibodies, to determine whether the constitutively active upstream kinases could phosphorylate endogenous p38. Expression of active MKK3bE or MKK6bE alone caused a strong phosphorylation of p38 above that seen in GFP-transfected cells (Figure 5-6).

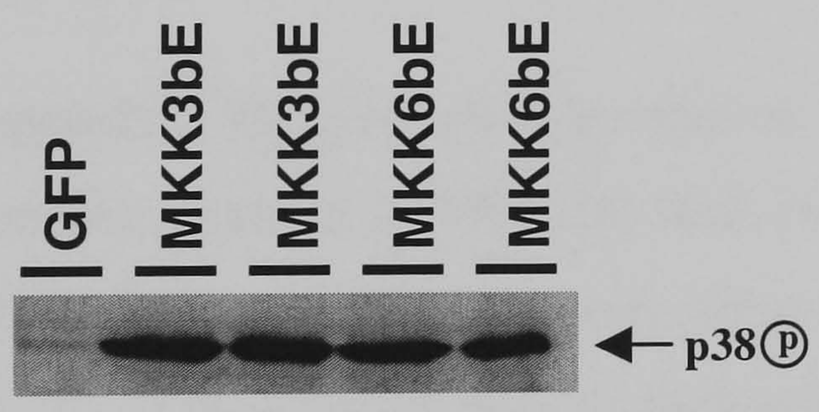

Figure 5-6: Activation of endogenous p38 by constitutively active mitogen-activated protein kinase kinase (MKK) mutants.
Myocytes were infected with adenoviral constructs encoding MKK3bE and MKK6bE, constitutively active mutants of MKK3b and 6b. 48 hours following transfection, cell were harvested and constituent protein probed with anti-phospho-p38 antibodies to detect p38 phosphorylation. p38 was strongly phosphorylated to a similar extent in both MKK3bE and MKK6bE expressing cells.

To determine whether this p38 phosphorylation was isoform selective, the experiment was repeated in the presence of wild type p38α and β. Under these conditions, p38α and β were strongly phosphorylated in the presence of either MKK3bE or MKK6bE (Figure 5-7), suggesting no isoform selectivity.

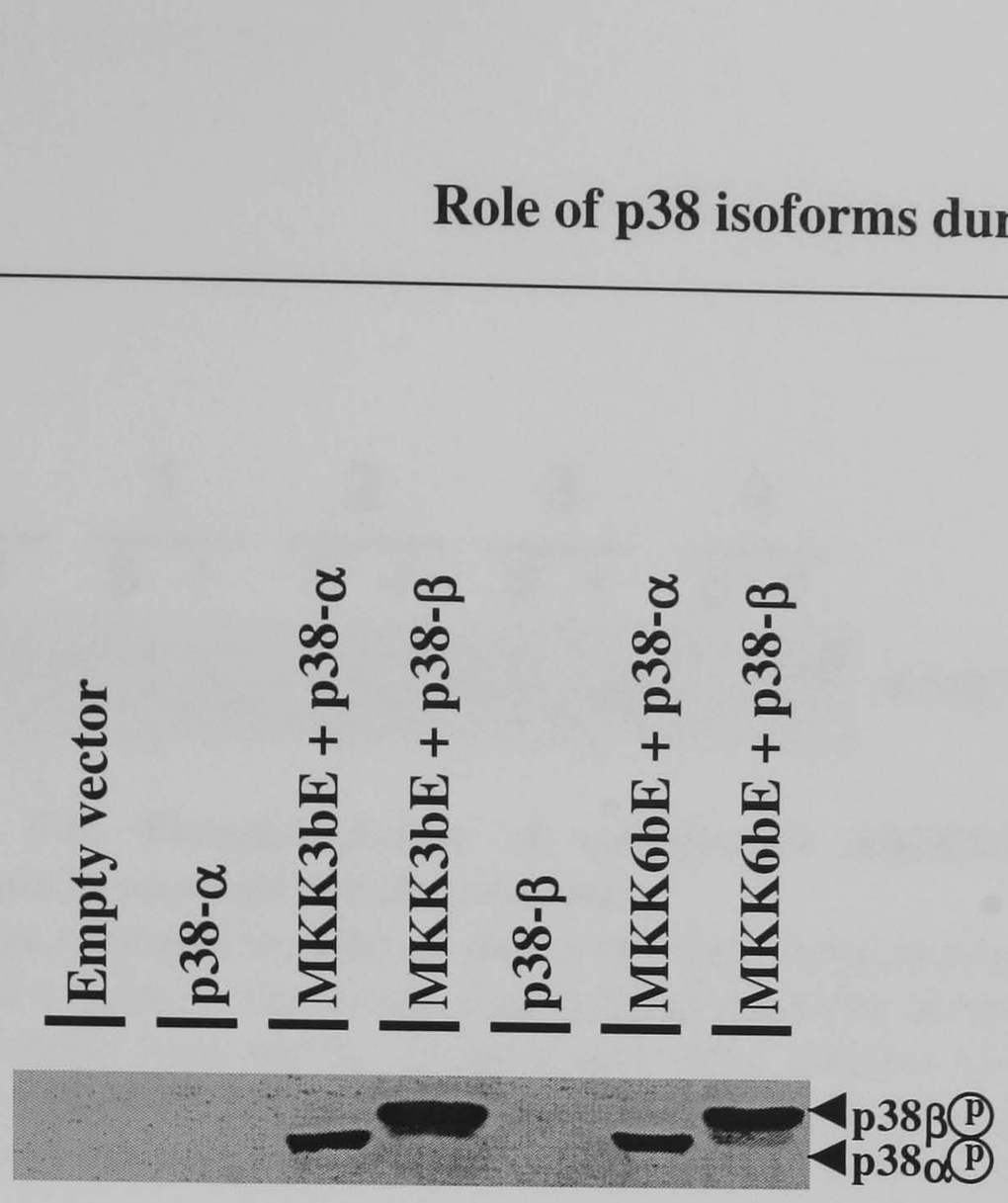

Figure 5-7: Activation of ectopically expressed p38α and p38β by constitutively active MKK3 or MKK6.
Myocytes were infected with adenoviral constructs encoding either MKK3bE or MKK6bE in combination with either p38α or p38β. 48 hours following transfection, cells were harvested and constituent protein probed with anti-phospho-p38 antibodies to detect p38 phosphorylation. Co-transfection with upstream MKKs caused a strong phosphorylation of p38α or β above baseline. There was no selectivity in the phosphorylation of p38 isoforms with either MKK3 or MKK6.

3.5 The activation of MKK3/6 during simulated ischaemia

If MKK3/6 were responsible for p38 phosphorylation during ischaemia then we should see a corresponding increase in MKK3/6 dual-phosphorylation prior to p38 phosphorylation during ischaemia. Myocytes were subjected to different durations of simulated ischaemia (1, 2, 3, or 4 hours) prior to harvesting. Constituent protein was then fractionated into soluble and insoluble fractions (using the protocol described previously; see Chapter 3 section 2.5). The high sequence homology around the phosphorylation site of MKK3 and 6 prevents the development of isoform-specific phospho-specific antibodies, therefore fractionated protein was Western blotted for phospho-MKK3/6. Figure 5-8 shows that MKK3/6 are phosphorylated between 2 and 3 hours ischaemia, which is identical to the time of p38 phosphorylation in this model (Figure 4-5, page 126). Interestingly, while phosphorylated MKK3/6 is exclusively in the soluble fraction prior to ischaemia, it is localised to the insoluble fraction after 2 hours simulated ischaemia. This may represent translocation of MKK3/6, or an upstream activator, during ischaemia.

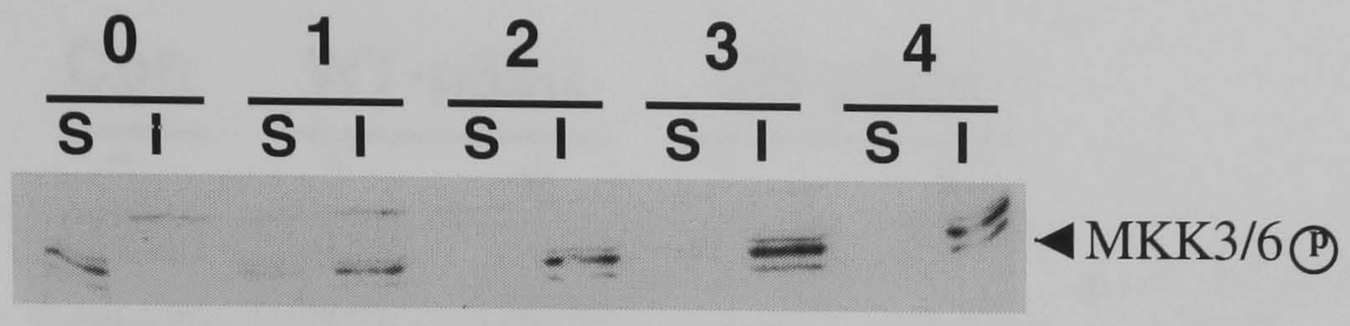

Figure 5-8: Phosphorylation of endogenous MKK3/6 during ischaemia in neonatal cardiac myocytes.
Myocytes subjected to different durations of simulated ischaemia (0, 1, 2, 3, and 4 hours) were harvested and protein separated into soluble (S) and insoluble fractions (I). Samples were then Western blotted with anti-phospho-MKK3/6 antibodies. MKK3/6 becomes phosphorylated exclusively in the insoluble fraction after 2 hours simulated ischaemia.

3.6 Effect of dominant negative p38 isoforms on cell viability following ischaemia

As shown in Figure 4-9 and by others (131, 133), inhibition of p38 during ischaemia using SB203580 protects against cell death. Since we demonstrate a selective activation of p38α over p38β during ischaemia, we would expect that protection ensues as a result of p38α inhibition with SB203580. To test this hypothesis we transfected cells with a dominant negative p38α mutant (residues 180-182; TGY to AGF; described previously (359)). As shown in Figure 5-9, 48 hours after transfection of either wild type or dominant negative p38α (DN-p38α), comparable overexpression of p38 can be detected (*Panel B*). Following 2.5 hours ischaemia, which is the time of maximal p38 activation, cells expressing DN-p38α showed no significant p38 activation, whereas cells expressing wild type p38α show increased p38 activation (Figure 5-9, *Panel A*).

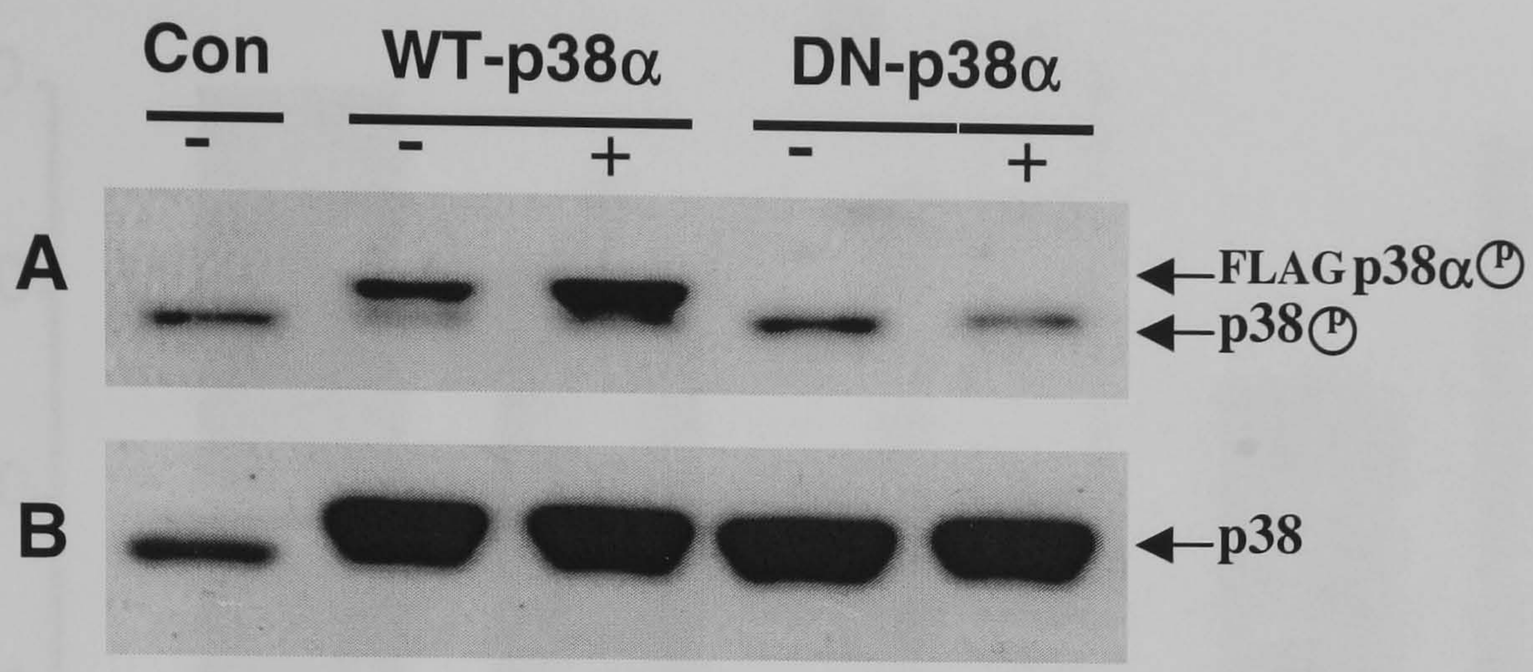

Figure 5-9: Modulation of ischaemic p38 activation with dominant negative-p38α.
Cardiocytes were infected with adenoviral constructs encoding FLAG-tagged dominant negative-p38α (DN-p38α) or wild type-p38α (WT-p38α). 48 hours post-infection, cells were subjected to 2.5 hours simulated ischaemia (+) or left untreated (-). Myocytes were then harvested and lysed for Western blot analysis. ***Panel A,*** activation of p38 was assessed using phospho-specific p38 antibodies. Ischaemia caused a marked activation of p38 in wild type-p38α transfected cells, whereas p38 activation decreased during ischaemia in cells expressing dominant negative p38α. ***Panel B,*** the total p38 levels were assessed with anti-p38 antibodies. Adenoviral infection causes a consistent level of expression of either WT- or DN-p38α, which is unaltered during ischaemia.

Parallel cells, infected identically, were subjected to 6 hours simulated ischaemia and 2 hours reperfusion to examine the effect of dominant negative p38α (DN-p38α) on cell viability following ischaemia/re-oxygenation. The cells expressing DN-p38α were protected against simulated ischaemia compared to empty vector-transfected cells (CK release = 82.9±3.9% and MTT bioreduction = 130.2±6.5%, n=8, $p<0.05$; see Figure 5-10). Expression of wild type p38α had no effect on cell injury (results not shown).

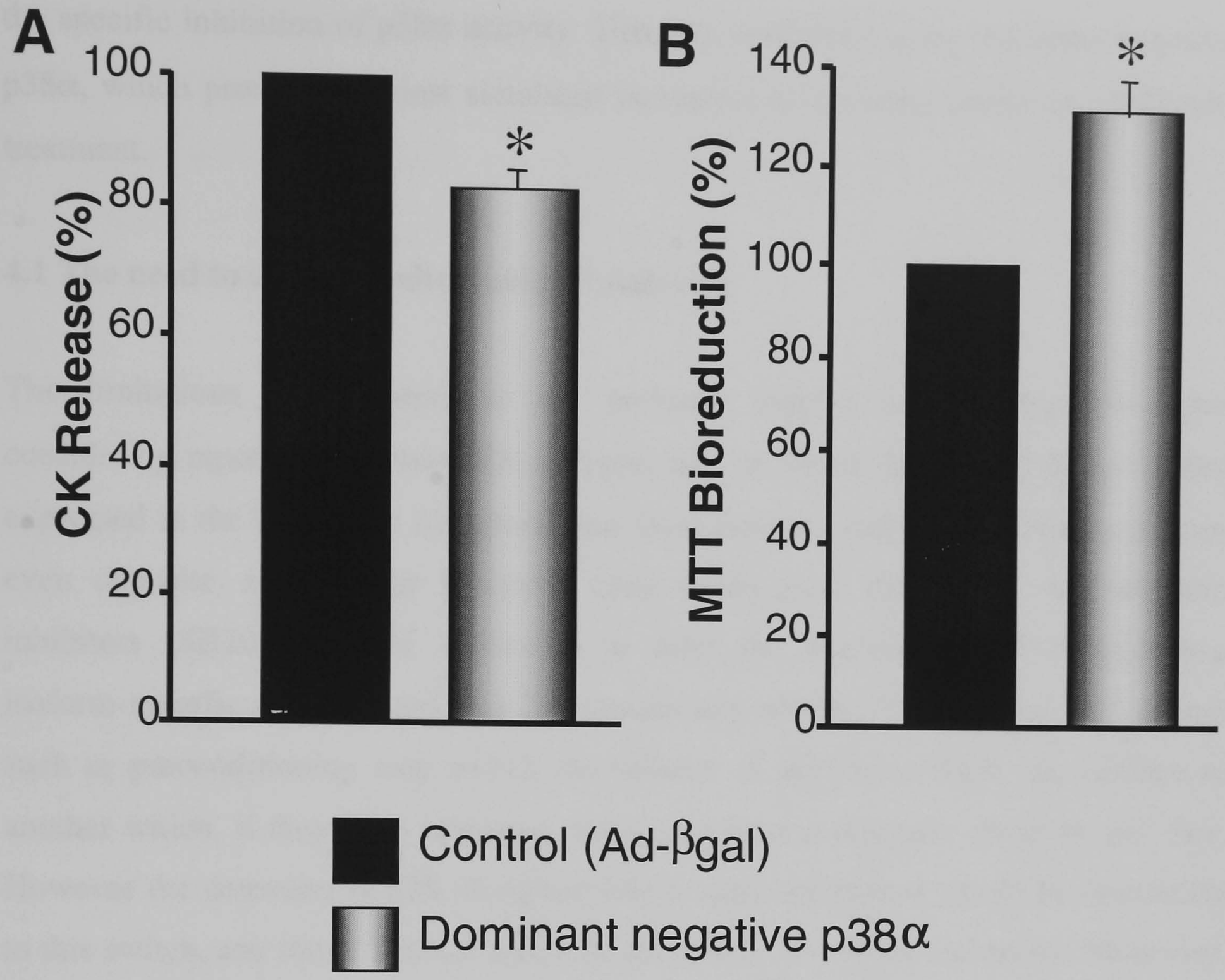

Figure 5-10: Effect of p38α activation on cell survival during ischaemia.
Myocytes, transfected as in Figure 5-9, were exposed to simulated ischaemia for 6 hours and the supernatant collected to assay CK leakage. ***Panel A,*** therefore represents cell injury during ischaemia alone. ***Panel B,*** cell viability was measured by MTT bioreduction within monolayers after 6 hours simulated ischaemia and 2 hours re-oxygenation. Injury in the presence of DN-p38α is expressed as a percentage of injury in the presence of an empty vector. Viability in cells treated with DN-p38α alone for 6 hours (without ischaemia) was equivalent to that observed in untreated cells (results not shown). Specific inhibition of p38α during ischaemia decreased cell injury and enhanced survival. All *p* values are for comparisons between ischaemia in the presence of DN-p38α v empty vector. *p<0.05, n=8.

4 Discussion

These studies demonstrate that p38α is the endogenous p38 isoform activated during simulated ischaemia in neonatal rat cardiac myocytes. In contrast, p38β is inhibited below basal levels during ischaemia. Since, as shown in the previous chapter, p38α and β inhibition with SB203580 protects against ischaemia, this can be attributed to

the specific inhibition of p38α activity. This was confirmed using dominant negative p38α, which protected against simulated ischaemia to a similar extent as SB203580 treatment.

4.1 The need to address individual p38 isoforms

The limitations of the work in the previous chapter became apparent when considering reports of various p38 isotypes, two of which (p38α and β) are highly expressed in the heart. The likelihood that these isotypes carry out different, perhaps even opposite, intracellular functions casts doubt over the use of non-selective inhibitors (SB203580) and antibodies to infer the function of p38. Neglecting isoform-specific effects could lead to contradictory results. For example, a treatment such as preconditioning may switch the balance of activation from one isoform to another which, if they have opposing roles, may have a dramatic effect on cell fate. However the detection of p38 phosphorylation using antibodies would be insensitive to this switch, and thus not show a significant change in overall activation. Moreover, inhibition of p38, when the major isoform activated is α may have significantly different results to inhibition when β is predominantly phosphorylated. This may explain the controversial findings in rabbit cardiomyocytes were SB203580 given during ischaemia actually accelerates injury (128). Therefore a greater insight into the differential activation of p38 isoforms allows a more complete appraisal of their effects on cell viability.

4.2 A differential role for p38 isoforms

To examine isoform-specific activation we used adenoviral-mediated expression of wild type p38α and β. Assessment of isotype activation after 2.5 hours ischaemia showed that p38α was activated, whereas p38β was significantly inhibited (Figure 5-2). These results were also confirmed in untransfected cells, using immunoprecipitated phospho-p38 (Figure 5-3). According to Wang and co-workers, p38α activation in neonatal cardiac myocytes is sufficient to cause apoptosis and cell

death, whereas increasing p38β phosphorylation promotes hypertrophy and survival (116). If this were true in our model, then the hypothesis would fit with a mechanism whereby p38α is the specific detrimental MAPK isoform activated by ischaemia. It is also possible that the inhibition of p38β contributes to injury, since activation of this isoform has been shown to promote survival (116).

Whilst p38 activation during ischaemia in this model is detrimental, others have shown that activation during preconditioning is beneficial, with SB203580 inhibiting protection (126, 130). Since p38 activation in these studies was simply measured using phospho-specific antibodies, it is unknown which isoform(s) is/are activated. It may be that the predominant isoform activated during preconditioning is p38β. If this were true, then this would provide an explanation for the opposite results to ischaemia, when alpha is predominantly phosphorylated. To examine this in our model we examined the phosphorylation of p38α and β during preconditioning. We observed a similar activation of p38α during preconditioning, although interestingly p38β remained phosphorylated. This does not account for the apparent dichotomous effects of preconditioning and lethal ischaemia, although the maintenance of p38β phosphorylation during preconditioning, as opposed to the inhibition seen during ischaemia, may contribute to these discrepancies.

4.3 The basis of differential activation of p38 isoforms

Understanding the cause of differential p38 isoform activation would aid the design of therapeutic strategies to protect the heart by preventing p38α activation during ischaemia. Theoretically, p38 isoforms could be activated by different upstream activators (or at least display differing selectivity), which may lead to specific isoform activation during ischaemia. Therefore we attempted to examine the activation of p38α and β by the known upstream activators MKK3 and 6. Specific activation of MKK3b and 6b was achieved by the adenoviral-mediated expression of constitutively active mutants, rendered constitutively active by a mutation of a Serine and Threonine residue to Glutamate (MKK3bE and MKK6bE). The acidic side chain on Glutamate

mimics the phosphorylation of these residues that occurs when they are activated endogenously. Co-transfection of these mutants with p38α or β showed that, under these conditions, either kinase was capable of phosphorylating both p38 isoforms.

These data were in disagreement with earlier studies from Davis' group examining the selectivity of MKK3 and 6 for p38α and β, which showed that whilst MKK6 was non-selective, MKK3 only phosphorylated p38α (357). Initially we presumed the discrepancy was due to a lack of selectivity following overexpression. Since this study (357) however, the Davis group have further characterised the interaction between MKK3/6 and p38α/β. They found that whilst MKK3 is unable to phosphorylate p38β, its splice variant MKK3b can (358). By constructing chimeric MKKs they mapped a p38-docking site to the N-terminal region of MKK3b, MKK6, and MKK6b, which is absent in MKK3. Therefore it is still possible that MKK3 is selectively activated during ischaemia in this model, which causes preferential activation of p38α.

4.4 Activation of MKKs during simulated ischaemia

Examining endogenous MKK3/6, we noted a strong phosphorylation after 2 hours simulated ischaemia (Figure 5-8), which suggests this causes the subsequent p38 activation during ischaemia. Unfortunately, the phospho-specific antibody was unable to distinguish between MKK3 and MKK6. It would be interesting to immunoprecipitate the active MKK3/6 and probe with the respective MKK3 or MKK6 antibodies, to determine whether ischaemia does selectively activate a particular MKK isoform.

Another feature of ischaemic MKK3/6 activation was the striking redistribution of phosphorylated MKK3/6 from soluble to insoluble fraction. It is unknown, from these results alone, whether this represents a relocalisation of total MKK3/6 or phosphorylated MKK3/6. In fact, it may simply be representative of a translocation of the upstream MKK3/6 activator from cytosol under basal conditions to insoluble fraction during ischaemia. Regardless of the mechanism for translocation, the

different localisation of upstream activators of p38 in control or ischaemic samples provides an intriguing possibility for isoform selectivity. If p38 isoforms (α and β) are present in different cellular fractions then isoform-selective activation could be induced by translocation of common upstream activators (i.e. MKK3/6). For example, if p38β is cytosolic and p38α in the insoluble fraction, then the localisation of MKK3/6 that we observe (i.e. soluble in controls and insoluble during ischaemia) may cause p38β phosphorylation basally, but p38α phosphorylation during ischaemia. Future studies examining the localisation of p38α and β should be designed to address this hypothesis.

4.5 The effect of preconditioning on isoform activation during ischaemia

In the previous chapter we showed that in preconditioned myocytes p38 phosphorylation during ischaemia was significantly diminished. Presumably, this is due to inhibition of endogenous p38α activation, since this is the isoform activated during ischaemia. Therefore, to verify this we examined the effect of preconditioning on ectopically expressed p38α and β. We observed a specific reduction in ischaemia-induced p38α phosphorylation following preconditioning, although p38β activation was unaltered. This selectivity for p38α inhibition may however, simply be a reflection of the lack of p38β phosphorylation during ischaemia.

4.6 The effect of p38-isoform activation?

The data presented within the previous chapter regarding protection following p38 inhibition during ischaemia relies on the use of the pharmacological inhibitor SB203580. Whilst SB203580 does inhibit p38 (Figure 4-8), it is also known to affect a range of other kinases. For example, it inhibits JNK (344), and PDK1 activity (345), whilst it activates Raf-1 (346). These non-specific effects of SB203580 complicate the interpretation of data for these and other (131-133) studies. Therefore we adopted a complementary approach by expressing a dominant negative p38α mutant in myocytes prior to ischaemia. The p38α was rendered dominant negative by

substitution of the Tyrosine and Threonine residues to non-phosphorylatable amino acids (residues 180-182; TGY to AGF). The effect of this dominant negative was to reduce p38 phosphorylation during ischaemia and protect. These data confirm the SB203580 results from the previous chapter and indicate that p38α activation alone is detrimental to myocyte survival during ischaemia.

4.7 Critique of methods

When examining the selectivity of MKK3/6 for p38α/β, constitutively active MKK3bE and MKK6bE mutants were used. Since completion of these studies it has become clear that MKK3b does not preferentially phosphorylate p38α, but MKK3 does (see section 4.3). Therefore, the experiment should be repeated with MKK3E mutants in combination with p38α or p38β to determine their selectivity in this model. In relation to ischaemia, although total MKK3/6 activation is increased, MKK3 and MKK6 phosphorylation should be quantified independently to determine whether a particular isoform is activated which may selectively phosphorylate p38α.

While inhibition of p38α protected cells during ischaemia, it would be advantageous to determine whether there is a reciprocal decrease in viability following p38α activation during ischaemia. Constitutively active MKK3 could be co-expressed with p38α prior to ischaemia, although the activation of p38α for hours prior to ischaemia would complicate data interpretation. Similarly, the effect of p38β activation during ischaemia was not determined in this model.

4.8 Conclusions

The phosphorylation of p38 isoforms during simulated ischaemia in this model is isoform selective, with p38α being activated. Moreover, p38β phosphorylation is suppressed below basal levels during ischaemia. Ischaemic preconditioning, which inhibits endogenous p38 activation, also specifically inhibits p38α phosphorylation

during ischaemia. This inhibition probably contributes to protection, since specific p38α inhibition with a dominant negative mutant confers a similar level of protection.

The cause of differential p38 activation is unknown, although it may be related to the activation of MKK3/6 seen during ischaemia. MKK3/6 is activated in the insoluble fraction during ischaemia, compared to its phosphorylation in the soluble fraction under basal conditions. Thus, MKK3/6 may co-localise with specific p38 isoforms under different physiological conditions. Alternatively, MKK3 and MKK6 may be differentially phosphorylated during ischaemia, which could dictate the pattern of p38 isoform activation.

Chapter 6. Ischaemic preconditioning in PKCε deficient mice

1 INTRODUCTION

The activation of either PKCδ (281) or PKCε (109) has been shown to protect the whole heart. However, PKCε activation may be more directly relevant to ischaemic preconditioning, since isoform-specific inhibitory peptides are able to abolish "preconditioning-like" protection in isolated neonatal rat (107) and adult rabbit (108) cardiomyocytes (108). These peptides function by preventing the binding of specific PKC isoforms to their respective receptors for activated C-kinase (RACKs). The rationale is that they prevent a specific PKC isoform from localising with its substrate(s), thus causing a loss of function. The difficulty in measuring isoform activation makes evaluation of the selectivity and efficacy of these peptides complex. Moreover, the simulated ischaemia in isolated cell models is only a substitute for ischaemia in the whole heart. Therefore, ischaemic preconditioning in the whole heart may depend on the activation of other PKC isoforms. There is therefore a need to further clarify the role of individual isoforms during ischaemic preconditioning in the whole heart. The lack of suitable pharmacological inhibitors, and the low efficiency of transfection *in vivo*, limits the models available to address isoform-specific PKC activation.

The ability to manipulate the mouse genome in a targeted and predictable fashion has enabled scientists to generate mutant mouse strains, which have been used to provide new and critical insights into the molecular mechanisms governing normal and disrupted biological processes. By using gene targeting via homologous recombination in murine embryonic stem cells, a specific gene can be either ablated or modified, and the mouse carrying the targeted locus can be generated for subsequent studies. To utilise these molecular genetic approaches in the field of cardiac physiology, models to assess whole organ function would be needed to analyse important aspects of any resultant phenotypes. Thus, techniques were developed in which the well-characterised isolated perfused heart apparatus was miniaturized such that parameters of myocardial contractility could be reproducibly assessed by using either a Langendorff or left-sided working heart preparation (360).

The Langendorff model involves retrograde perfusion of the heart with buffer, which enters the coronary arteries via the coronary sinuses at the base of the aortic root. This enables the heart to contract and remain viable for many hours. Ischaemia can be mimicked by clamping the aortic inflow tubing, thus preventing buffer-perfusion. Ischaemia/reperfusion injury has been characterised using this isolated mouse heart model (361), and preconditioning can be mimicked by brief cycles of ischaemia/reperfusion prior to lethal global ischaemia (29, 232).

We obtained mice lacking PKCε protein due to a targeted disruption of the *pkc-ε* alleles. We therefore sought to use these mice to investigate ischaemic preconditioning, in an attempt to delineate the role of this PKC isoform in protection following preconditioning in the mouse.

2 Specific Methods

2.1 Principles of transgenic mice

The generation of transgenic mice is a multi-stage procedure that requires the generation, amplification and purification of a suitable transgene cassette inserted into a plasmid vector. The expression of the transgene is confirmed *in vitro* by transfection into eukaryotic cells. The transgene is then introduced into the embryonic stem (ES) cell either by vector transfection and selection or by direct microinjection of plasmid-free transgene fragments into embryonic pronuclei. Manipulated embryos can then be placed in the reproductive tract of a pseudo-pregnant recipient or introduced into the embryo of another mouse strain by injection into the blastocyst stage embryo. The genomic DNA of live-born pups is then analysed for the presence of the transgene DNA sequence. Littermates, heterozygous for the transgene, can then be mated to produce homozygous offspring. Below is an example of a targeting strategy involving pronuclear microinjection (Figure 6-1).

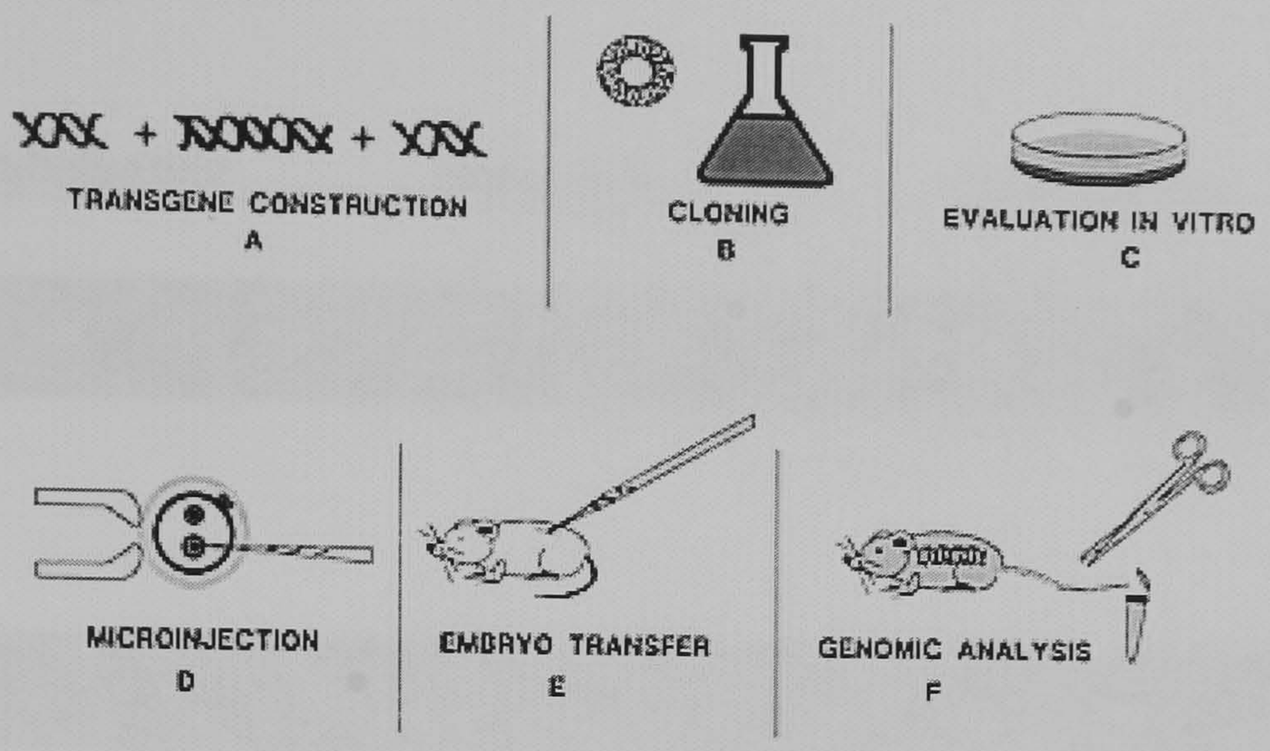

Figure 6-1: Sequence of events in the generation of a transgenic animal by pronuclear microinjection.
Panel A, the double-stranded DNA components of the transgene are combined enzymatically to yield a transgene expression cassette. ***Panel B,*** transgene cassettes are inserted into plasmid vectors and cloned. ***Panel C,*** transgene-bearing plasmids are transfected into cultured eukaryotic cells to evaluate expression of the transgene. ***Panel D,*** plasmid-free transgene fragments are introduced directly into embryonic pronuclei. ***Panel E,*** manipulated embryos are placed in the reproductive tract of a pseudo-pregnant recipient. ***Panel F,*** the genomic DNA of live-born pups is analysed for the presence of the transgene DNA sequence.

2.2 Generation of PKC epsilon deficient mice

Dr Daniel Pennington at the Imperial Cancer Research Fund, London, carried out the targeting of the *pkc-ε* allele and the generation of *pkc-ε* homozygous mutant mice. In brief, a 9.3Kb EcoRI fragment containing the first exon of the *pkc-ε* gene was isolated from a murine 129/Sv genomic DNA library (gift from T. Rabbits, LMB, Cambridge). To generate a targeting vector, a positive selectable cassette was introduced into the PstI site of exon 1. This cassette contains stop codons in all three frames, an independent ribosomal entry site (IRES) followed by the *LacZ* gene with an SV40 polyadenylation sequence, and a neomycin phosphotransferase gene (MC1Neo poly(A); gift from A. Smith, Centre for Genome Research, Edinburgh). Correctly targeted GK129 ES cells were selected and then injected into C57BL/6 blastocysts. Chimeric mice were bred to C57BL/6 mice in specific pathogen free (SPF) conditions and offspring crossed to generate *pkc-ε* homozygous mutant animals. PKCε deficient mice were shown to be negative for PKCε protein by Western Blotting.

2.3 Experimental protocols

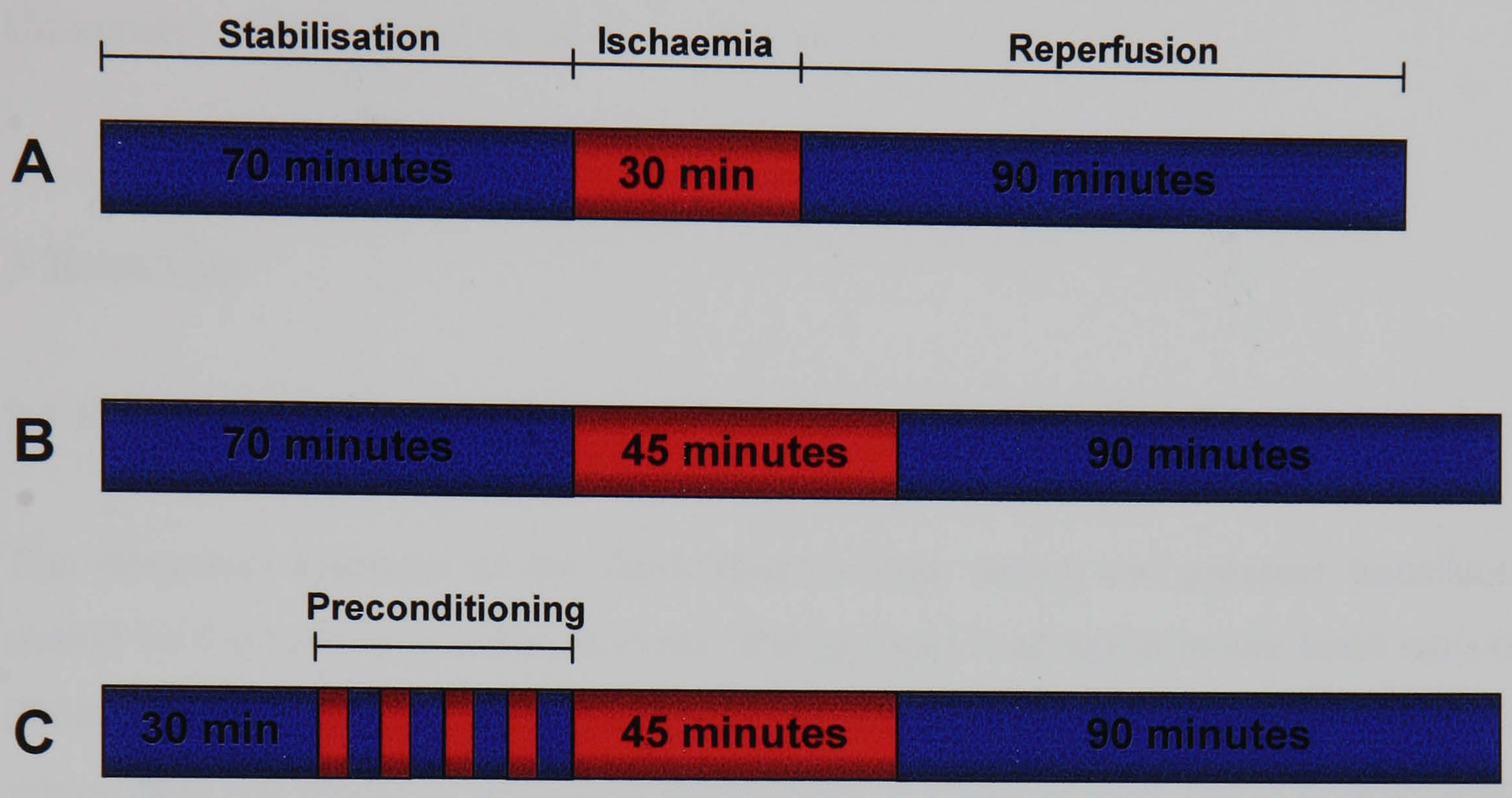

Figure 6-2: Schematical representation of mouse heart perfusion protocols.
Two different durations of ischaemia were used, 30 minutes (*Panel A*) and 45 minutes (*Panel B*). Preconditioning was simulated with 4 cycles of 4 minutes ischaemia and 6 minutes reperfusion before 45 minutes index ischaemia (*Panel C*). A 90 minute duration of reperfusion was used since this was sufficient to allow maximal contractile recovery and washout of dehydrogenases, NADH and other co-factors from the necrotic tissue that may cause false positive tetrazolium staining of dead cells.

2.4 Western blot analysis

Samples were obtained from the liver or whole heart by homogenisation in 2×SB. Western blotting was then carried out, as described previously, to assess PKC isoform abundance in mice wild type (+/+), heterozygous (+/-), and homozygous (-/-) for a disruption within the *pkc-ε* alleles. All PKC antibodies were murine monoclonal antibodies (Transduction Laboratories, UK), which were detected using a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (DAKO A/S, Denmark).

2.5 Statistical analysis

Data for functional recovery at the end of reperfusion were analysed by one-way ANOVA, followed were appropriate by the Tukey-Kramer test for pair-wise comparisons. Linear regression was carried out using SigmaStat statistical package.

Comparisons of infarct size with respect to total myocardial volume between groups were made by analysis of covariance (ANCOVA) using an Excel plug-in (Ferris State University). A probability value of ≤ 0.05 was considered significant.

3 RESULTS

3.1 Frequency-response characteristics of isovolumic fluid-filled balloons

The frequency response of the fluid-filled balloon, tubing and pressure transducer should be flat to at least 10Hz, to avoid false pressure readings at mouse heart rates of 580bpm used in these studies. The frequency response characteristics of the balloon, tubing, and transducer to sinusoidal fluctuations in pressure were flat to at least 30Hz (Figure 6-3).

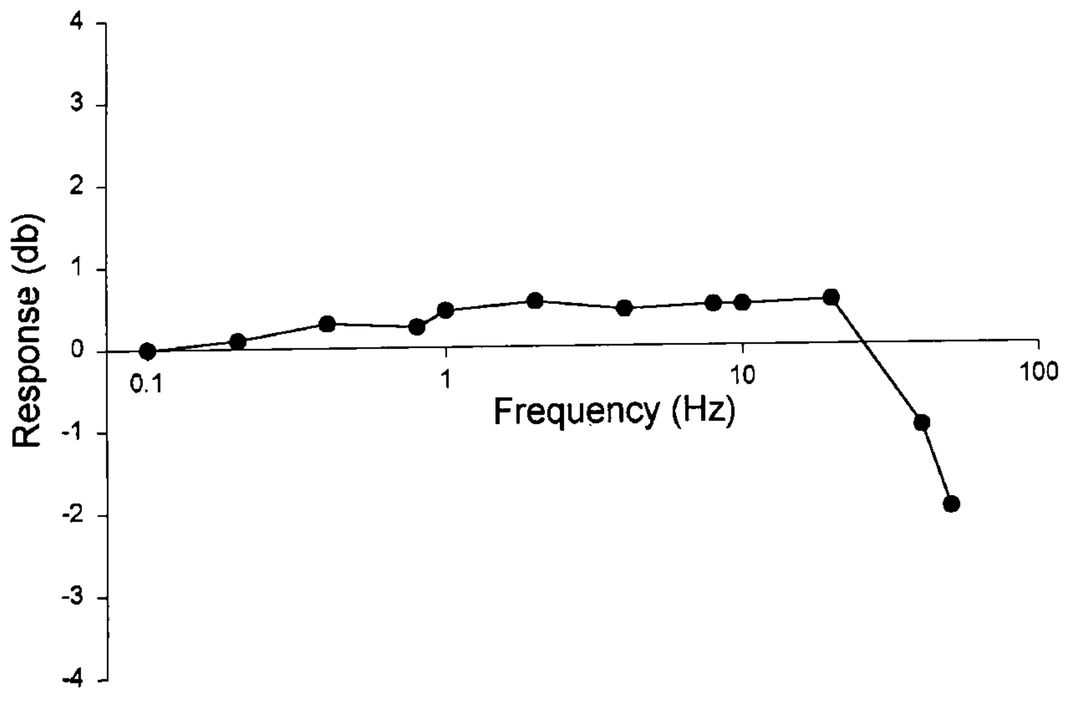

Figure 6-3: Typical frequency-response trace for a fluid-filled balloon and coupling tubing.

3.2 Preliminary studies on global ischaemia and reperfusion

Preconditioning has been proposed to delay the onset and rate of necrosis during index ischaemia. We chose 45 minutes as the duration of lethal ischaemia since in a

standard inbred strain (T/O mice, B&K Universal Ltd, England) infarction was significantly larger than after 30 minutes (26.3±3.7% v 8.0±2.7%, $p<0.05$, and see Figure 6-4). Since we were able to detect this difference, and preconditioning is thought to delay infarction by 10-15 minutes, we reasoned that preconditioning prior to 45 minutes ischaemia would be detectable as a reduction in infarct size.

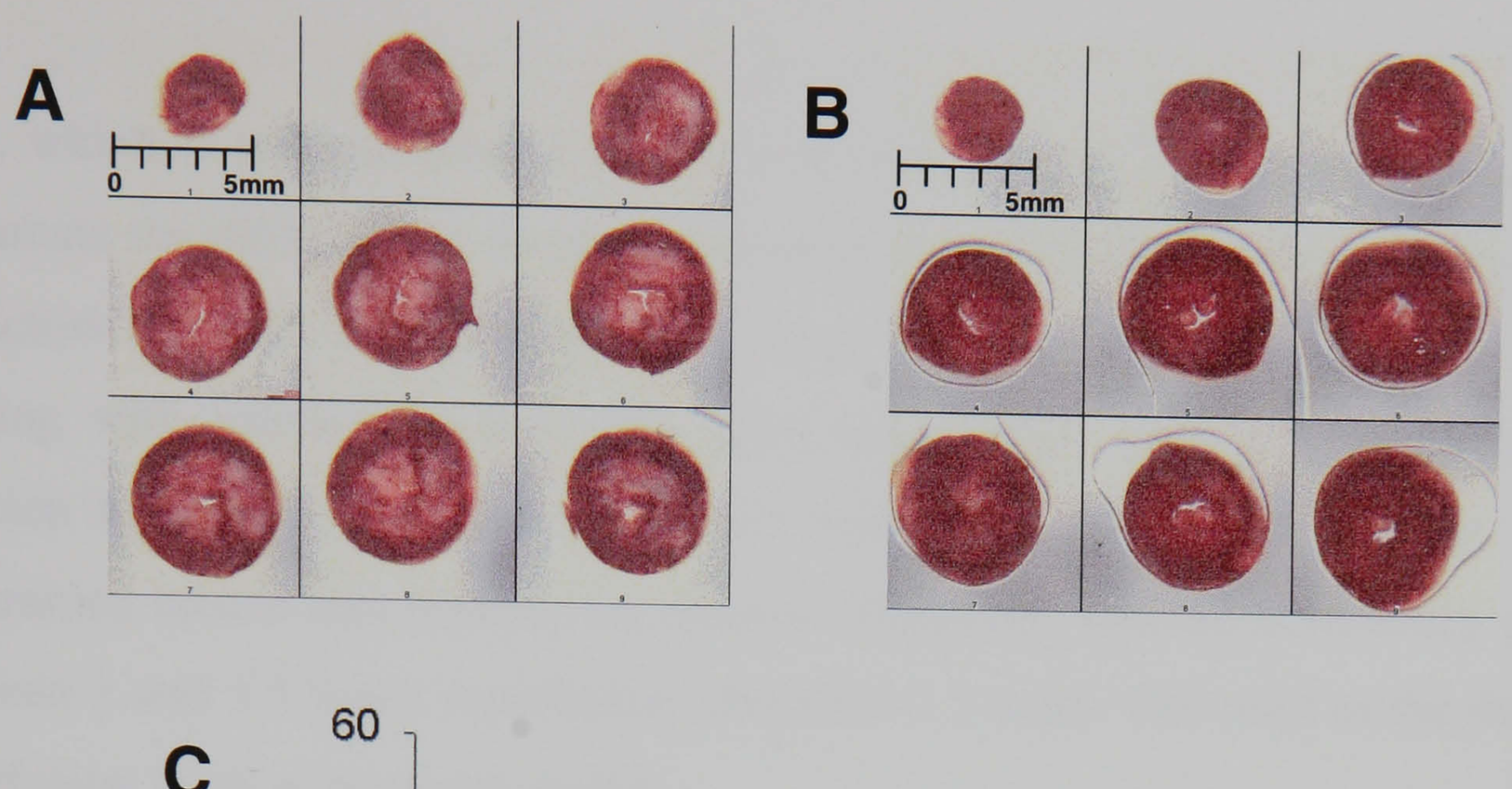

Figure 6-4: The effect of ischaemic duration on infarct size.
Panels A and B, typical mouse hearts sectioned after triphenyl-tetrazolium chloride (TTC) staining to demarcate infarction (pale tissue) after 45 minutes (*Panel A*) or 30 minutes (*Panel B*) of global no-flow ischaemia and 90 minutes reperfusion. Actual infarct volumes as a percentage of heart volume are 24.2% and 4.1% respectively. ***Panel C,*** mean infarct size following 45 or 30 minutes ischaemia and 90 minutes reperfusion. *p<0.05.

Yellon's group have previously shown that 4 cycles of 5 minutes ischaemia/5 minutes reperfusion preconditions the isolated mouse heart (232). Initial experiments in our model showed that 5 minutes ischaemia caused a slight elevation in end diastolic pressure and predisposed hearts to dysrhythmias during subsequent reperfusions. We therefore used 4 × 4 minutes ischaemia/6 minutes reperfusion, which caused no observable detrimental effects (results not shown). Moreover, extending reperfusion to 2.5 hours after this preconditioning stimulus caused no perturbation in left ventricular developed pressure (LVDP) compared to aerobically perfused controls.

TTC, which is reduced from a yellow to a red pigment in living cells, was used to demarcate the infarct zone. Insufficient washout of dehydrogenases, NADH and other co-factors from the necrotic tissue may cause false positive staining of dead cells. In keeping with previous studies (26), pilot experiments showed that increasing the duration of reperfusion from 1.5 to 2.5 hours had no significant effect on TTC-determined infarct size (results not shown). Moreover, functional recovery plateaued between 1 and 1.5 hours reperfusion, therefore 1.5 hours was used as the duration of reperfusion in all subsequent studies.

3.3 Preconditioning studies in a standard inbred mouse strain

A total of 33 male T/O mice were randomised to either control (no preconditioning) or ischaemic preconditioning (4 × 4 minutes ischaemia/6 minutes reperfusion) groups. Four hearts were excluded during the stabilisation period due to high aortic flows (2), low developed pressure (1), or persistent dysrhythmias (1). There were no significant differences in baseline parameters between groups (Table 6-1).

Experimental Groups	**Control** (n=15)	**IP** (n=14)
Body Weight (g)	35.0±0.8	35.8±0.6
Wet Heart Weight (mg)	132.3±5.5	133.1±5.2
Heart Volume (mm^3)	95.6±4.2	97.6±3.6
Coronary Flow (ml/min)	2.5±0.1	2.4±0.1
Isolation Time (sec)	154±6	152±5
LVDP (mmHg)	70.3±1.6	69.2±1.9

Table 6-1: Morphometric characteristics and baseline parameters of isolated buffer-perfused hearts from T/O mice.
Values are mean±SEM. There were no statistically significant differences between the groups for any parameters. Coronary flow and LVDP were recorded after 30 minutes stabilisation. Isolation time refers to the time interval between thoracotomy and retrograde perfusion.

Preconditioning prior to the index ischaemia reduced mean infarct size and improved contractile recovery following subsequent reperfusion (Figure 6-5). These data demonstrate that infarct size is halved in preconditioned hearts and contractile recovery is enhanced.

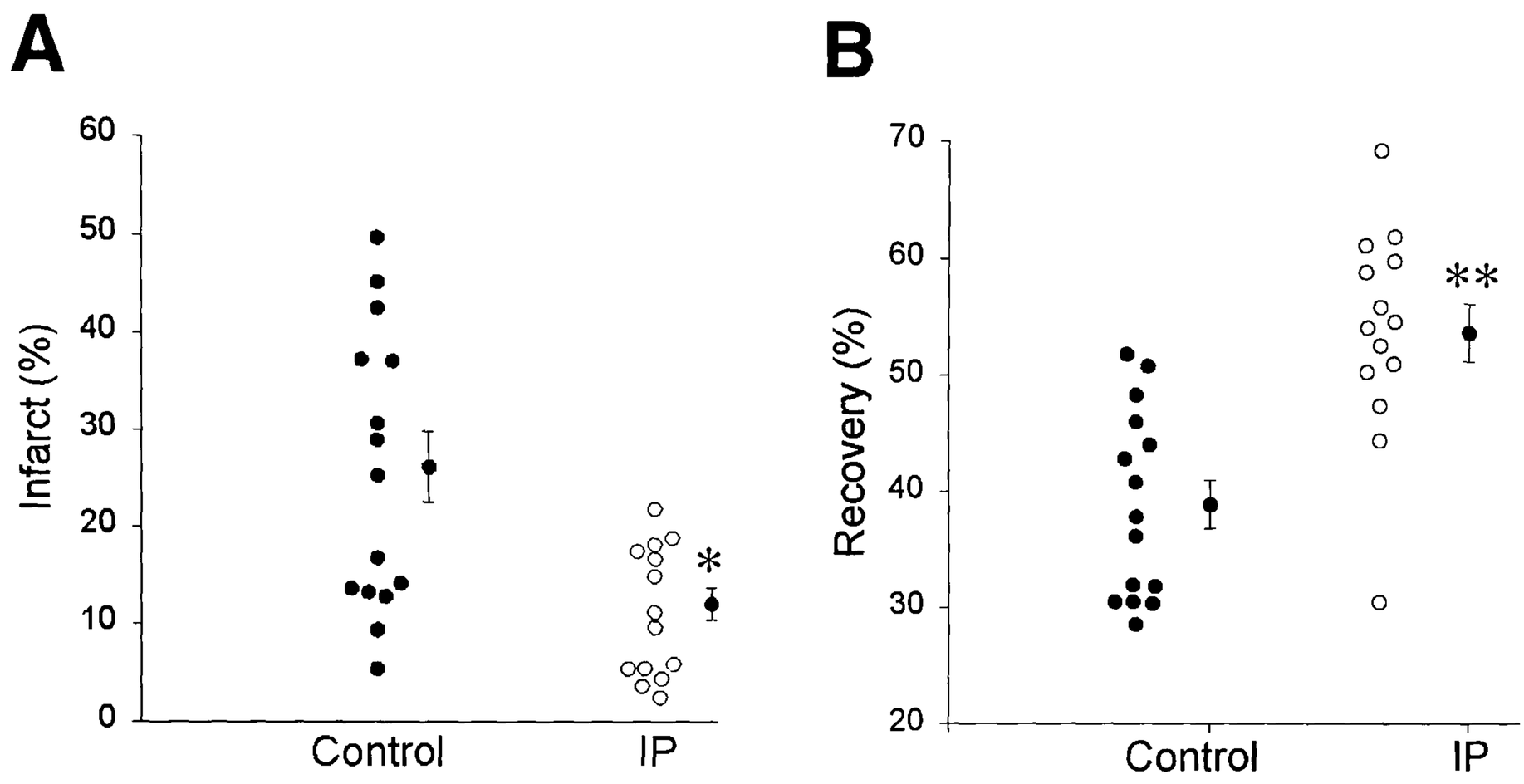

Figure 6-5: Effect of ischaemic preconditioning (IP) on infarct size in buffer perfused hearts from T/O mice.
Panel A, mean data for infarct volume as a percentage of total heart volume in control (●, n=15) and IP (○, n=14) groups (*p<0.05). In the control group infarction was 26.3±3.7%, whereas infarction in the IP group is 12.2±1.7%. ***Panel B,*** contractile recovery in control and IP hearts expressed as a percentage of baseline left ventricular developed pressure (**p<0.001). Data shows that preconditioning significantly decreases infarct size and improves functional recovery.

There was however, a large variability in infarct size within the controls, ranging from 5-50% (Figure 6-5, *Panel A*). The net effect of this variability is the need for large experimental numbers to reach statistical significance. When the infarct volumes are expressed against the respective heart volumes we see a close positive correlation (Figure 6-6, r=0.96 and 0.92 for control and preconditioned groups respectively). When analysed (using analysis of covariance [ANCOVA]) with respect to total myocardial volume, the reduction in infarction in preconditioned hearts reached a higher level of significance (p<0.001, see Figure 6-6).

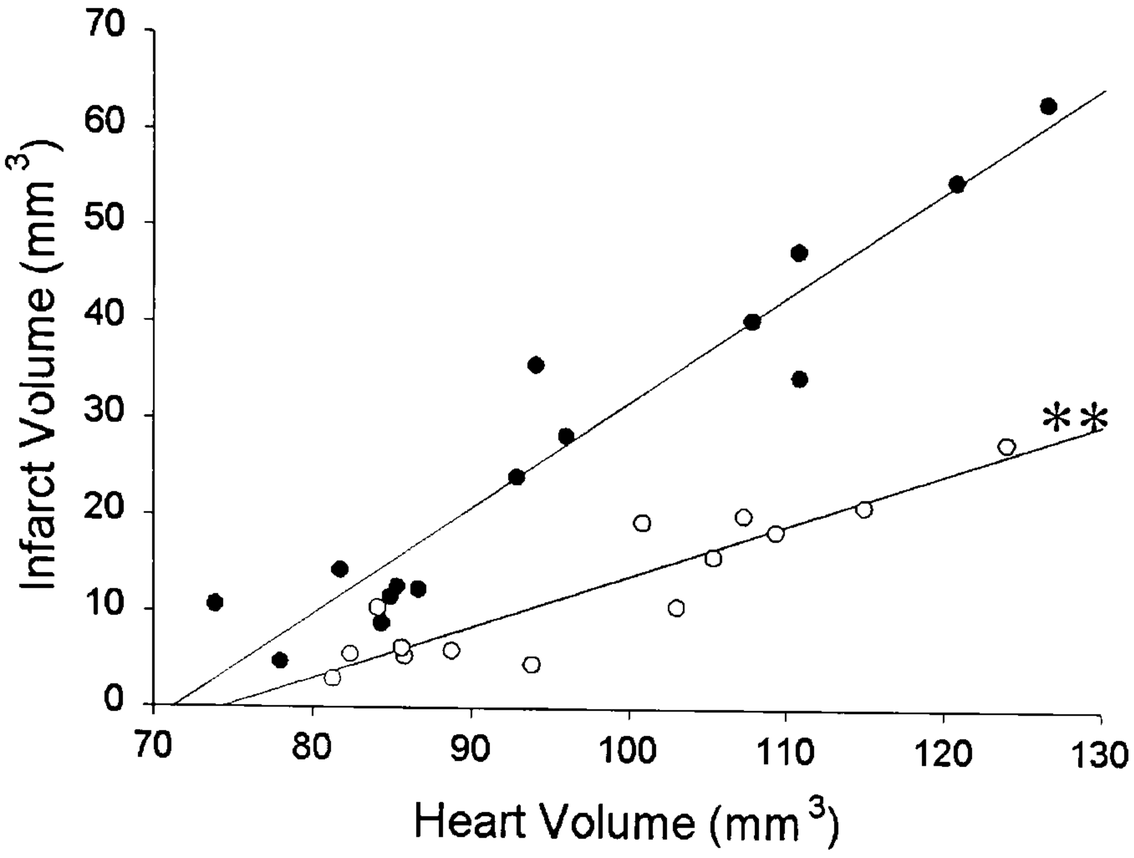

Figure 6-6: Infarct volumes expressed against heart volumes in control and preconditioned hearts.
Infarct volume in control (●) and IP (○) hearts expressed with respect to total heart volume. Linear regression for control (r=0.96) and IP (r=0.92) groups both reached statistical significance ($p<0.001$). Data shows that, for a given heart volume, the infarct volume is less in ischaemically preconditioned hearts. **$p<0.001$, by ANCOVA.

3.4 Verification of transgenic genotype

Offspring of heterozygous (+/-) × knockout (-/-) breeding pairs were randomised to either preconditioning or control groups. Since the genotype of the progeny would be predicted to be 50% heterozygous and 50% knockout, tail clips were simply retained at –20°C for genotyping after all the physiological data had been collected. Purified DNA from the tail clips was subjected to PCR with oligonucleotide primers specific for sequences both 3' and 5' of the inserted cassette. Thus, in mice homozygous for a disruption within the PKCε alleles (-/-), the PCR product was a large 700bp fragment containing the cassette. In wild type (+/+) mice a smaller 350bp fragment was obtained, whereas heterozygous mice (+/-) yielded both fragments (Figure 6-7).

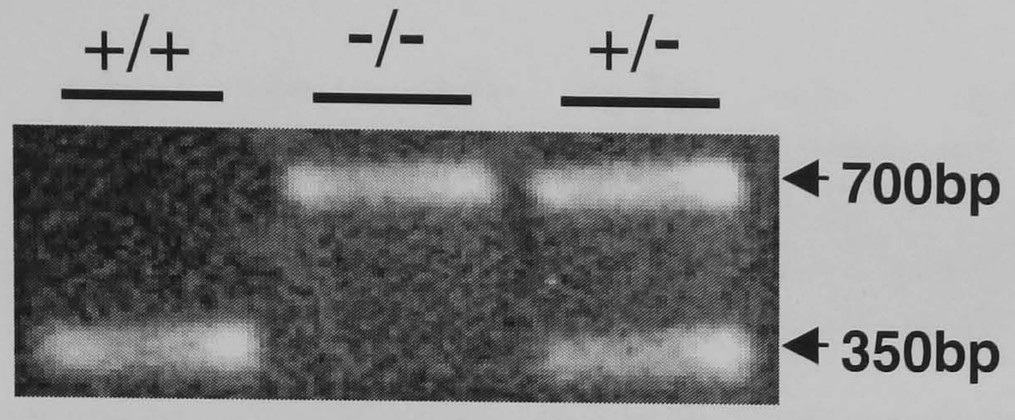

Figure 6-7: Polymerase chain reaction (PCR) used to identify genotype in transgenic mice.
Wild type (+/+) mice yield a fragment encoding 350bp, whereas knockout (-/-) mice generate a larger 700bp fragment, and heterozygous (+/-) mice produce both fragments.

3.5 Analysis of PKC isoform expression levels in transgenic mice

Mouse hearts were homogenised and constituent protein subjected to Western blot analysis with anti-PKC antibodies. PKCε protein was detected in both wild type and heterozygous mice, although PKCε was absent in knockout mice (Figure 6-8). A major concern of knockout technologies is the possibility of developmental upregulation of closely related isoforms to compensate for the lack of a particular gene. Therefore, heart protein was probed with antibodies for PKCδ and α, two other PKC isoforms implicated in myocardial protection. As shown in Figure 6-8 the levels of PKCδ and α remain unaltered between groups.

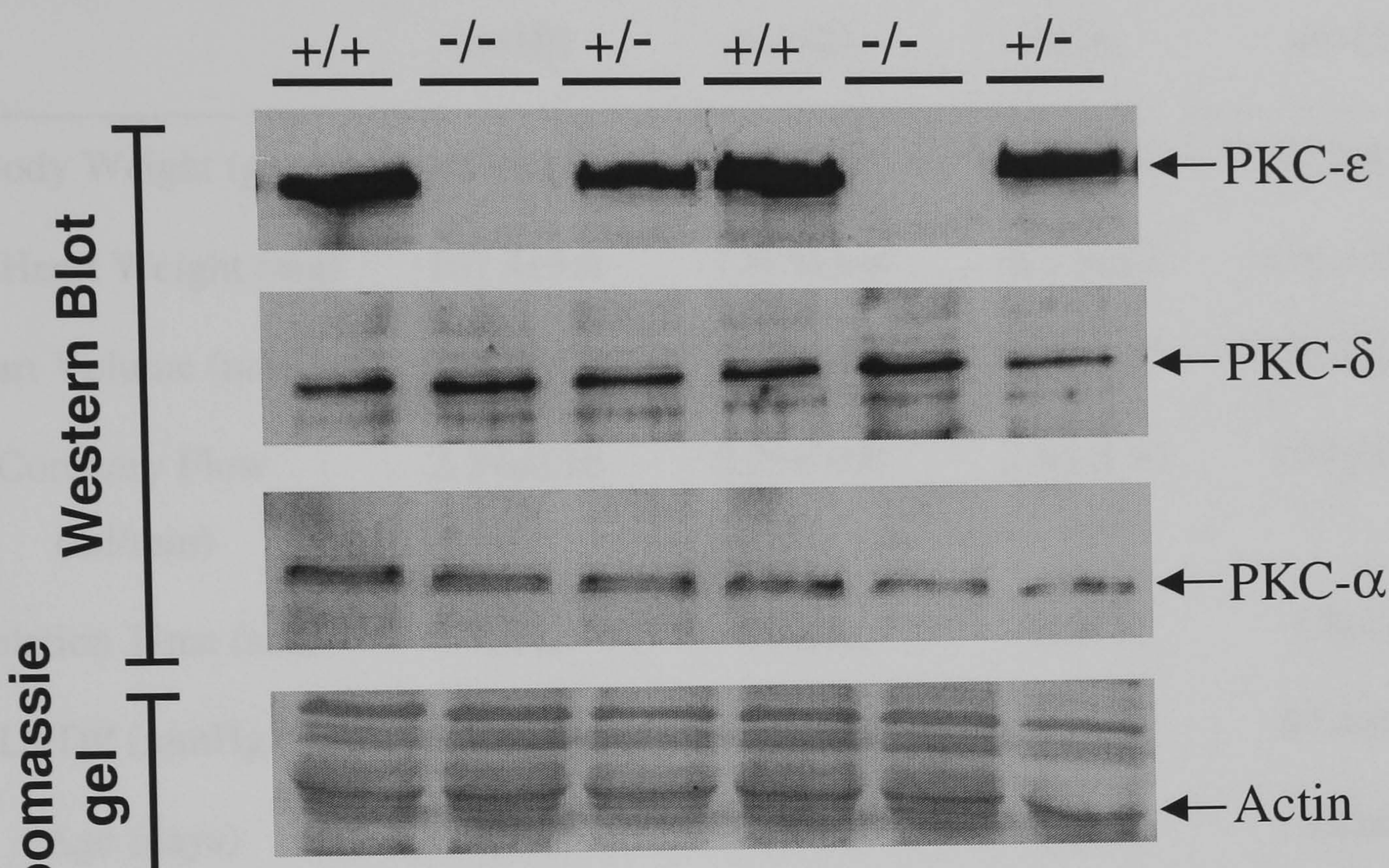

Figure 6-8: Analysis of PKC isoform expression in hearts from mice wild type (+/+), heterozygous (+/-), and homozygous (-/-) for a disruption within the *pkc-ε* alleles.
Panel A, Hearts from (-/-) mice have no detectable expression of PKCε protein, whereas hearts from (+/+) and (+/-) mice show similar levels of PKCε protein expression. PKCε genotype has no significant effect on expression of PKCδ or PKCα, two other PKC isoforms implicated in IP. Equal protein loading was confirmed by coomassie staining of identically loaded gels. There was no difference in actin levels between groups.

3.6 Preconditioning studies in PKC epsilon deficient mice

Having characterised the protein expression in transgenic mice, we then sought to examine the effect of ischaemic preconditioning in these mice. A total of 48 male mice entered the study, but five hearts were excluded during stabilisation due to high aortic flows (2), low developed pressure (1), or persistent dysrhythmia (2). There were no significant differences in baseline parameters between groups (Table 6-2).

Experimental Groups	Control (+/-) (n=10)	IP (+/-) (n=12)	Control (-/-) (n=8)	IP (-/-) (n=13)
Body Weight (g)	41.4±1.9	40.7±1.7	38.0±1.4	41.9±1.2
Wet Heart Weight (mg)	167.4±9.0	170.7±5.6	152.3±5.9	154.1±4.8
Heart Volume (mm^3)	123.9±5.5	125.8±5.9	115.2±4.7	120.8±2.9
Coronary Flow (ml/min)	2.39±0.18	2.35±0.06	2.36±0.10	2.34±0.10
Isolation Time (sec)	137±8	144±15	158±12	130±10
LVDP (mmHg)	69.6±1.9	67.6±0.9	71.9±2.2	67.4±1.4
Age (days)	121±3	122±3	119±2	123±2

Table 6-2: Morphometric characteristics and baseline parameters of isolated buffer-perfused hearts from mice heterozygous (+/-) and homozygous (-/-) for a disruption within the *pkc-ε* alleles.

Values are mean±SEM. There were no statistically significant differences between the groups for any parameters. Coronary flow and LVDP were recorded after 30 minutes stabilisation. Isolation time refers to the time interval between thoracotomy and retrograde perfusion.

In heterozygous (+/-) mice, which contain the full complement of PKCε protein, preconditioning resulted in a significant reduction in infarct size (Figure 6-9, *Panel A*; $p<0.05$), although again this reached higher significance when expressed against heart volume (Figure 6-9, *Panel B*; $p<0.001$).

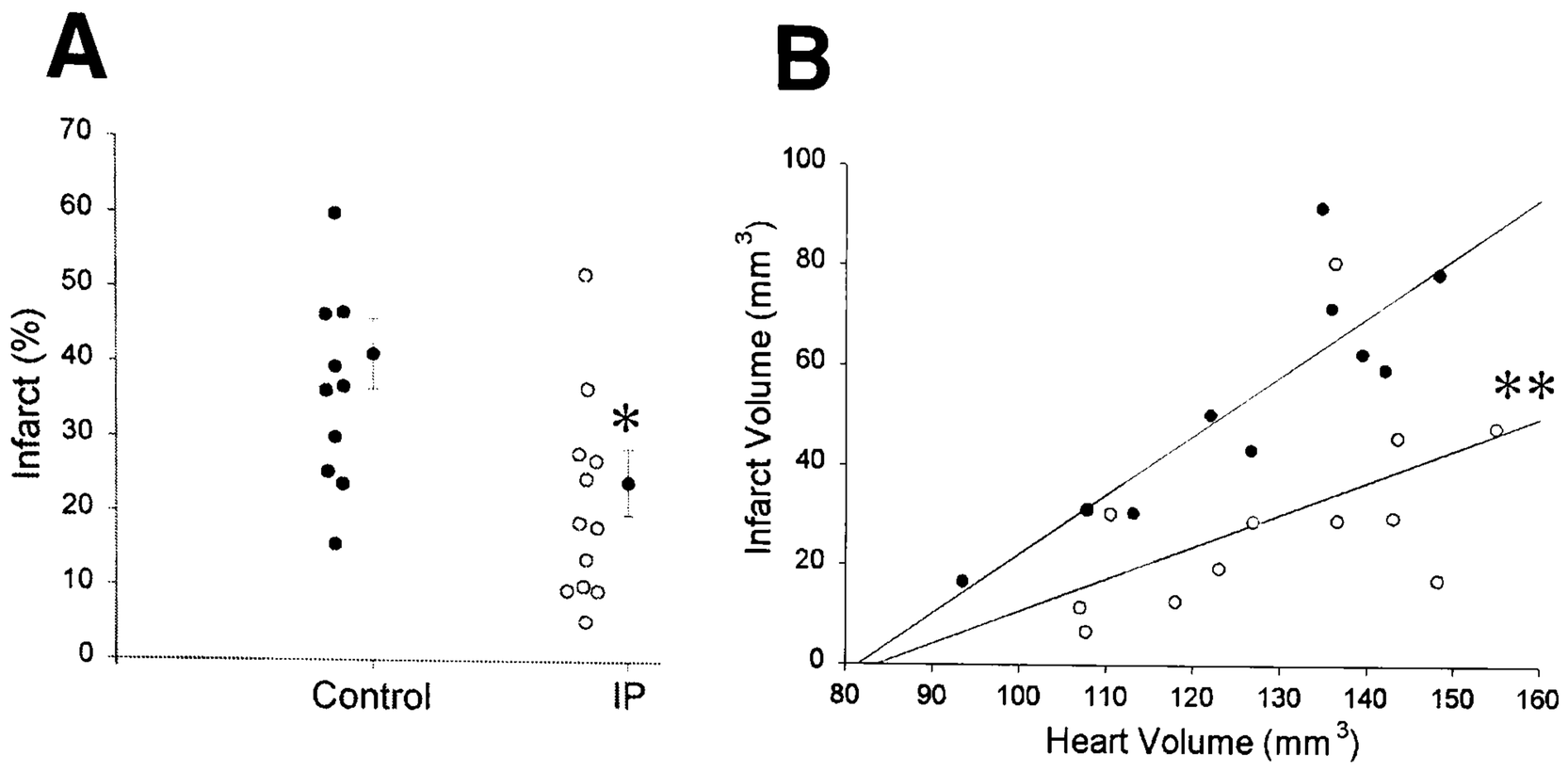

Figure 6-9: The effect of ischaemic preconditioning (IP) in isolated buffer-perfused hearts from mice heterozygous (+/-) for a disruption within the *pkc-ε* alleles.
Panel A, mean infarct volume, expressed as a percentage of total myocardial volume, was significantly lower in preconditioned hearts compared to controls (24.3±4.5% v 41.3±4.7%, *p<0.05 [ANOVA]). ***Panel B,*** infarct volume in control (●) and IP (○) hearts from (+/-) mice expressed against total heart volume. Preconditioning prior to ischaemia significantly reduced infarct volume compared to controls (**p<0.001 [ANCOVA]).

In knockout (-/-) hearts that lack the *pkc-ε* gene and PKCε protein, there was no statistical difference in infarct size between ischaemic preconditioning and control hearts, when analysed as a percentage of (Figure 6-10, *Panel A*), or with respect to (Figure 6-10, *Panel B*), myocardial volume.

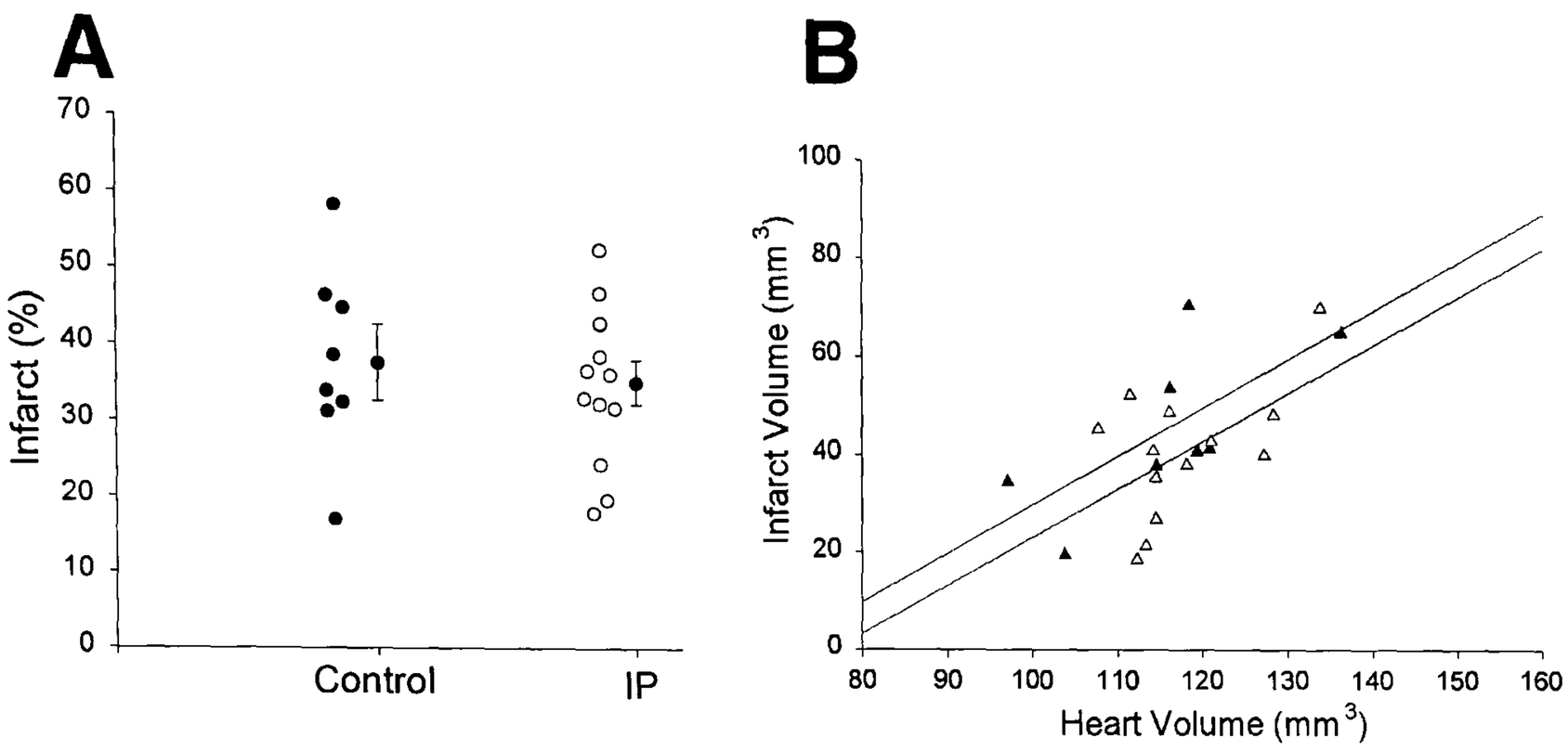

Figure 6-10: The effect of ischaemic preconditioning (IP) in isolated buffer-perfused hearts from mice homozygous (-/-) for a disruption within the *pkc-ε* alleles.
Panel A, mean infarct volume, expressed as a percentage of total myocardial volume, was unaltered in preconditioned hearts compared to controls (36.4±2.9% v 38.8±4.5%). ***Panel B,*** infarct volume in control (▲) and IP (△) hearts from (-/-) mice expressed against total heart volume. Preconditioning prior to ischaemia had no effect on infarct volume when assessed by ANCOVA.

By superimposing the infarct data from these two studies we can see that the relationship between myocardial volume and infarct size was identical in heterozygous (+/-) and knockout (-/-) control hearts, indicating that the lack of PKCε *per se* had no effect on infarct size following ischaemia/reperfusion (Figure 6-11). However, the lack of PKCε did prevent any effect of preconditioning on infarct size, since the only significantly different group is the preconditioned heterozygotes.

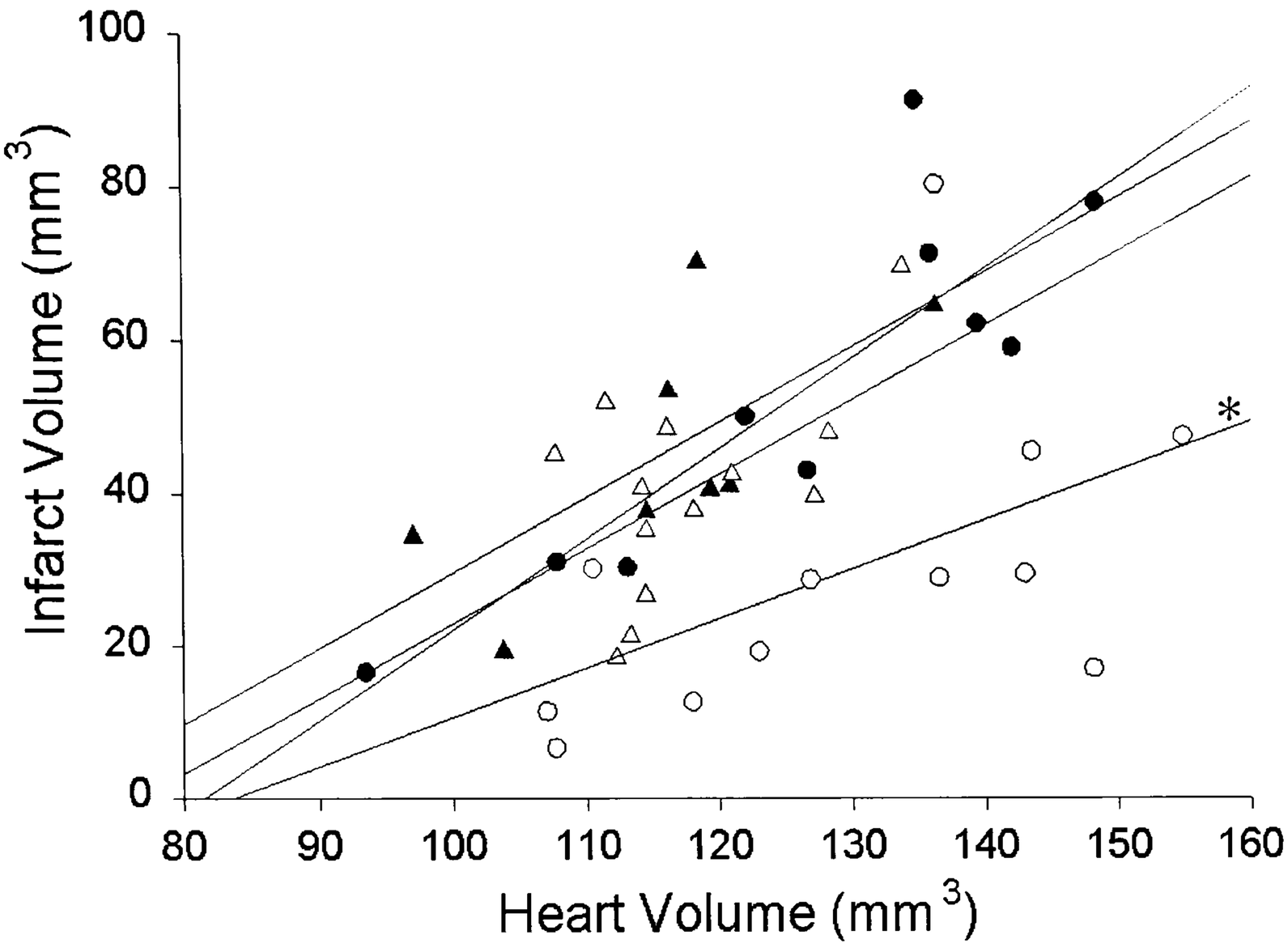

Figure 6-11: Infarct volume in control (filled) and IP (open) hearts from heterozygous (circles) and knockout (triangles) mice expressed against total heart volume.
There is no difference in infarct size in control (+/-), control (-/-), and IP (-/-) groups. The IP (+/-) hearts had significantly less infarction than all other groups (*$p<0.005$, by ANCOVA).

Although there was no benefit of preconditioning on infarct size in hearts from (-/-) mice, surprisingly contractile recovery was preserved in this group to a level similar to that seen in preconditioned (+/-) hearts (Figure 6-12).

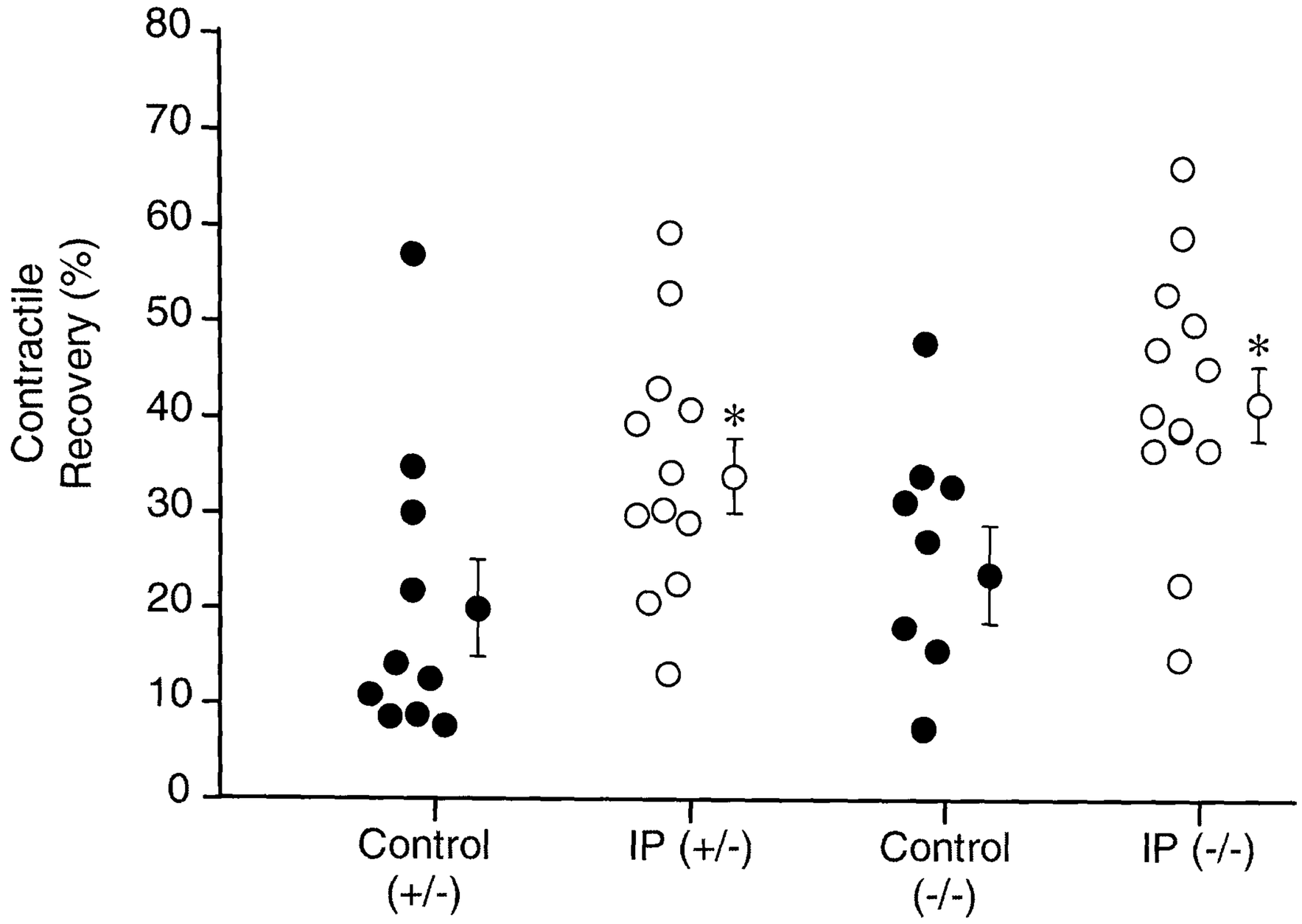

Figure 6-12: The effect of ischaemic preconditioning (IP) on contractile recovery in isolated buffer-perfused hearts from mice heterozygous (+/-) and homozygous (-/-) for a disrupted *pkc-ε* allele.
Mean data for contractile recovery in control (●) and IP (○) hearts from (+/-) and (-/-) mice. IP significantly improved recovery in hearts from both (+/-) and (-/-) mice. *p<0.05.

4 DISCUSSION

Using Langendorff-perfusion of hearts from a standard in-bred mouse strain we have shown that preconditioning with 4 × 4 minutes ischaemia/6 minutes reperfusion prior to 45 minutes global ischaemia improves contractile recovery and reduces infarct size. However the percentage of the heart volume that undergoes infarction is highly variable. The most significant factor contributing to this variability is heart size, with smaller hearts exhibiting disproportionately small infarct volumes. This effect can be unmasked by analysing the covariance between infarction volume and heart volume. In common with a standard inbred mouse strain, preconditioning reduces infarction in mice with a single disrupted *pkc-ε* allele that retains a significant cardiac PKCε complement. However in their littermates, with a similar genetic background but with both *pkc-ε* alleles disrupted and no cardiac PKCε protein, preconditioning fails to

reduce infarction. Paradoxically however, despite these dichotomous effects on infarction, preconditioning improves post-ischaemic contractile recovery in both the presence and the absence of cardiac PKCε protein. These data show that PKCε is essential for the reduction in infarct size but not for the increase in contractile recovery that follows preconditioning. This suggests that distinct intracellular mechanisms may underlie the different manifestations of preconditioning, cautioning extrapolation between models with diverse endpoints.

4.1 Relationship between heart volume and infarct volume

In this model, infarction volume is related to heart volume and weight. Thus when heart volume is less than $80mm^3$ (≈110mg) we found little or no infarction after 45 minutes global ischaemia. Superficially this seems surprising and counter-intuitive. However, identical relationships between volume at risk of infarction and final infarction volume are documented in better characterised small animal models of myocardial infarction (17, 55, 362). Arguably the best-characterised small animal model is regional myocardial ischaemia in the rabbit. In this model a number of studies have shown that infarction following 30 minutes of regional ischaemia is absent when the risk zone volume is less than $300mm^3$ (55, 362). In an identical manner to our study, these studies have also reported statistically significant effects of interventions based on comparisons of mean infarction:risk zone ratios. However these comparisons must be viewed as relatively crude since they ignore a critical determinant of infarction volume and assume a zero intercept. Previous reports of murine models of infarction have also ignored this contribution to the variance in infarction:risk zone ratios. It is possible therefore that the ANCOVA analysis reported in this manuscript would have allowed statistical significance to be achieved in these previous studies with fewer observations. This potential saving in resources underlies the importance of the observation. Table 6-3 displays the infarct size and heart weight derived from manuscripts that use an isolated mouse heart perfusion model and document both infarct size and heart weight data.

Reference	Ischaemic time (minutes)	Mean heart weight (mg)	Mean infarct size (%)
Sumeray *et al.*, 1998a (361)	20	190	10
Xi *et al.*, 1999a (363)	20	230	20
Xi *et al.*, 1998 (29)	20	244	24
Xi *et al.*, 1999b (364)	**20**	**254**	**27**
Yoshida *et al.*, 1996 (365)	30	140	8
Yoshida *et al.*, 1997 (366)	30	140	7
Sumeray *et al.*, 1998a (361)	**30**	**170**	**30**
Sumeray *et al.*, 1998b (232)	30	200	55
Saurin *et al.*, 2000	**45**	**128**	**26**
Greaves *et al.*, 2000	45	138	31
Saurin *et al.*, 2000	45	158	42

Table 6-3: Infarct size and heart weight of isolated mouse hearts subjected to varying durations of ischaemia in published studies
Data for Saurin *et al.* are derived from studies contained within this thesis. Greaves *et al.* are unpublished observations from our group.

These data indicate that for a given ischaemic duration infarct size increase with increasing heart size. Furthermore, when heart size decreases (bold values), the length of ischaemia must be increased to maintain the same level of infarction. These data suggest a disproportionately relationship between heart size and infarct size in isolated perfused mouse hearts.

The mechanism(s) by which risk zone volume influences susceptibility to infarction is unknown. Our presumed explanation for this effect is superfusion of nutrients and metabolites between heart and surrounding Krebs-Henseleit buffer in our experiments or between risk zone and normally perfused myocardium in the rabbit. Thus the value of the intercept, which provides the volume below which infarction is absent, reflects the surface area to volume ratio of the risk zone that is large enough to provide adequate superfusion to maintain myocyte viability during 45 minutes ischaemia. This could explain why this constant is lower in our studies where the risk zone is spherical compared to that in the rabbit where small risks zones are serpiginous.

4.2 Ischaemic preconditioning in isolated buffer-perfused mouse hearts

To our knowledge there have been only 2 previous reports of preconditioning in the isolated mouse heart (29, 232). Using one of these protocols, which consisted of 4 cycles of 5 minutes ischaemia and 5 minutes reperfusion, we found hearts were disposed to dysrhythmias and elevated diastolic pressures during preconditioning. It is possible that this duration of ischaemia was too severe, although viability assays were not performed following this preconditioning protocol. Instead the ischaemic duration was shortened to 4 minutes, and reperfusion increased to 6 minutes. This protocol prevented the adverse effects during preconditioning. We therefore characterised our own protocol for preconditioning with 4 cycles of 4 minutes ischaemia with 6 minutes of intervening reperfusions before 45 minutes global ischaemia. The endpoints used to assess myocardial injury were contractile recovery and infarct size, which were enhanced and attenuated respectively by ischaemic preconditioning.

4.3 The role of PKC isoforms in preconditioning

The difficulty in measuring the activity of individual PKC isoforms has lead to contradictory reports implicating different isoform(s) in ischaemic preconditioning. Using translocation as a surrogate for activation, it has been suggested that PKCα, δ, ε, and η are all activated following a preconditioning stimulus (284, 291). Until recently this was simply associative data, but the design of isoform specific peptides that inhibit PKC translocation has provided the strongest evidence in favour of PKCε in early ischaemic preconditioning. These studies, in isolated neonatal cardiac myocytes (107) and adult cardiomyocytes (108), show that preconditioning requires the translocation of PKCε for protection against simulated ischaemia. Moreover, peptides that function by promoting the translocation of PKCε have also been shown to protect both neonatal cardiac myocytes and the mouse heart against ischaemia (109). Furthermore, Ping *et al.* have showed that overexpression of PKCε protects adult cardiomyocytes against simulated ischaemia (223). A major concern in all these studies is the inability to accurately assess isoform activation. This is because

isoform-specific substrates are currently unknown and differing antibody affinities, isotype abundance, and *in-vitro* activation, complicates the interpretation of PKC activity after immunoprecipitation of individual isoforms. The requirement for some measure of activity is highlighted in the activator/inhibitor peptide studies, since these selective strategies rely on the premise that PKC translocation is synonymous with activation. This premise may not be correct with the possibility of non-translocation dependent activation (63), and even translocation dependent inhibition (298). The use of deficient mouse-lines is resistant to such advances in our understanding of PKC biology.

The other advantage of using specific PKC isoform deficient mouse lines is the ability to investigate the role of PKC isoforms in more physiological models of preconditioning. The difficulty in efficiently transfecting the whole heart has so far limited genetic approaches to cell-based models of simulated ischaemia. Furthermore, even in these models, other uncertainties exist since our group (221) has previously suggested that specific activation of PKCδ in rat myocytes protects against simulated ischaemia. In fact the downstream signalling pathways activated by PKCδ and ε, which are thought to lead to protection, have many similarities (18, 367). For these reasons we sought to examine ischaemic preconditioning in hearts from mice deficient in PKCε.

4.4 Ischaemic preconditioning in isolated hearts from *pkc-ε* knockout mice

For controls, we chose to use mice heterozygous (+/-) for a disrupted *pkc-ε* allele, rather than wild type (+/+) mice. This decision was reached after ensuring that mice with these genotypes had a similar complement of PKCε within their hearts (Figure 6-8). Thus all male offspring of (+/-) × (-/-) matings could be randomised before genotyping. This reduced colony maintenance costs and eliminated bias since genotypes were not determined until all physiological data had been collected. A further advantage of this approach is that mice share the same genetic background,

thus reducing the effect of modifier genes and thereby increasing the probability that the effects observed are due to the gene of interest.

We have shown that protection is present in heterozygous (+/-) mice, whereas the knockout (-/-) littermates lacking PKCε show no reduction in infarct size following ischaemic preconditioning. This demonstrates that preconditioning, at least in the mouse, protects against infarct size via a signalling pathway that involves the specific activation of PKCε. It is possible that selective activation of other PKC isoforms may be sufficient to protect in this model, but this does not detract from the fact that ischaemic preconditioning relies on the activation of the endogenous PKC-epsilon isoform. Future studies in this mouse line should address whether activation of PKC with PMA is sufficient to protect knockout hearts, thus determining whether the dependence on PKCε during preconditioning is a unique ability of this isoform to protect, or whether preconditioning only activates this endogenous isoform. If the latter is true, then future studies should not disregard the other PKC isoforms, since specific activation of these pharmacologically may offer an equal therapeutic benefit.

4.5 The dichotomy of contractile recovery and infarct size

In hearts lacking PKCε, although infarct size is unaltered by preconditioning, contractile recovery is still preserved. We are not able to provide a definite explanation for this dichotomy. However one can speculate that it is related to the mutually exclusive processes of stunning and infarction. For example, antioxidants given at the onset of reperfusion have been shown to improve contractile recovery with no effect on necrosis. Puett *et al.* (368) showed in dogs that oxypurinol, which inhibits xanthine oxidase and decreases free radical production, improved regional ventricular function when given during reperfusion following 60 minutes ischaemia, but failed to reduce infarct size. The authors suggested oxygen radicals contribute to stunning of reversibly damaged myocardium but not to the final extent of necrosis. Ischaemic preconditioning has also been shown to reduce oxygen radical production during reperfusion following ischaemia (262), it is therefore possible that this reduction in oxidant stress and consequential stunning is independent of PKCε

activation in the mouse heart. This would explain the improvement in functional recovery, but not infarction, in PKCε deficient hearts following preconditioning. Other evidence suggesting these processes differ in murine myocardium is that the attenuation of stunning following ischaemic preconditioning requires a greater ischaemic trigger than that for infarct size reduction. For example, using preconditioning cycles of 2 × 2.5 minutes ischaemia/2.5 minutes reperfusion, Xi *et al.* showed that ischaemic preconditioning reduces infarction but has no effect on contractile recovery in the isolated mouse heart (29). The fundamental question to answer however is whether contractile function is a marker of cell viability or whether it is a more indicative marker of contractile function within the viable population of cells. The fact that contractile function and infarction can be dissociated in these studies, and by others (262), argues in favour of the latter.

4.6 Critique of methods

A major concern of knockout technologies is the possibility that closely related genes may be developmentally upregulated to counteract the knockout phenotype. In this respect, although no significant changes in PKCα or PKCδ protein levels could be detected, it is still possible that these isoforms may counteract the knockout effect by binding to and activating PKCε-specific substrates. Furthermore, although PKCε displays a 10-fold greater affinity for its intracellular RACK (RACK1), this RACK is still capable of binding other isoforms (Dr Daria Mochly-Rosen, personal communication). Therefore, in the absence of PKCε, another isoform may translocate and bind to the PKCε RACK and stimulate "epsilon-like" responses. To further characterise these effects, PKC isoform localisation should be compared between heterozygous and knockout mice. Nevertheless, these concerns are, to an extent, alleviated by the lack of preconditioning in knockout mice.

Using heterozygous and knockout mice offers the advantage of a more homogenous genetic background, however it is still possible that PKCε protein levels are reduced in heterozygotes. Although this does not abolish preconditioning in our model, it may

limit the protection. For this reason, the level of protection seen in these hearts should be compared to protection in hearts from wild type mice. Alternatively, the levels of PKCε protein in heterozygous and wild type mice should be quantified by slot blotting.

Most isolated heart perfusion studies assess cell injury by enzyme (creatine kinase) leakage. This proved technically difficult in our model since the low coronary flow through mouse hearts necessitates that the heart and cannula are submersed in 37°C Krebs throughout the experiment. This prevents the easy collection of coronary effluent, although this could have been achieved by cannulation of the pulmonary artery.

4.7 Conclusions

We have used sibling mice of a single sex to demonstrate that disruption of both, but not one, *pkc-ε* alleles leads to the loss of cardiac PKCε protein and ischaemic preconditioning against infarction. The similarity of genetic background suggests the association between PKCε and preconditioning is causal. In murine myocardium, PKCε is necessary for the infarct size reduction that accompanies preconditioning. For selective activation of PKC to become a feasible clinical target, future work must focus on the design of isoform-specific PKC activators.

Chapter 7. General discussion and conclusions

1 RATIONALE FOR EXPERIMENTAL METHODS

Figure 7-1 shows a schematical representation of a signalling pathway that is initiated at a cell surface receptor and culminates in an intracellular effect.

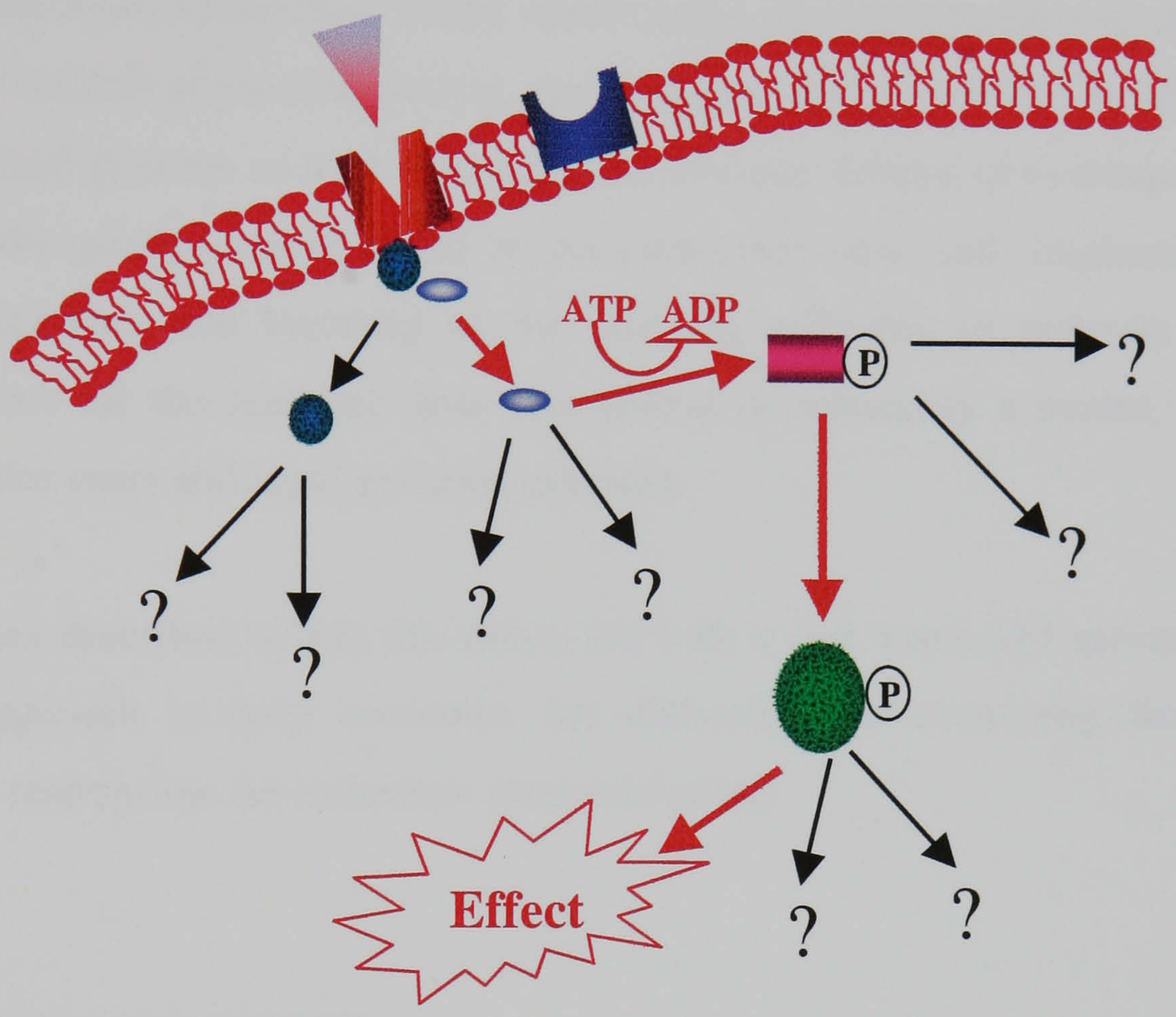

Figure 7-1: Schematical representation of an intracellular signalling pathway.

This over-simplified schematic highlights both the advantages and difficulties of investigating cell signalling pathways. If it were therapeutically desirable to pharmacologically agonise the above effect (i.e. ischaemic preconditioning), then agents that target distal molecules in the signalling pathway would be more specific. Thus, understanding the complete pathway may enable the design of pharmacological agents with fewer side effects. The technical difficulties in investigating these signalling pathways stem from the large number of structurally related proteins that constitute the intracellular protein kinases. Thus, unlike the relatively specific agonists/antagonists of cell surface receptors, the pharmacological tools available to manipulate signalling proteins are limited and often less specific.

To overcome these difficulties, one can adopt a genetic manipulation based approach, were the structure of a particular gene (i.e. kinase) is altered to produce an inhibitory or activating effect. To modify the gene of interest, DNA must be introduced into cells (by transfection) to allow the synthesis of a modified protein. Unfortunately, the poor transfection efficiencies in the whole heart, necessitates the use of surrogate, cell-based models of preconditioning. An alternative approach is the modification of a whole animal genome so a particular protein is either ablated or overexpressed. This involves the genetic manipulation of the embryonic stem cell, implantation into a female recipient, and breeding of the resulting offspring to generate an animal homozygous for the modified gene (the animal is commonly a mouse, due to low maintenance costs and rapid gestation periods).

The studies described within this thesis use both a cell based and genome-targeting mouse approach to help overcome the difficulties in examining the signalling pathways responsible for ischaemic preconditioning.

2 SUMMARY OF RESULTS

This thesis examines the role of protein kinases implicated in the intracellular signalling pathway responsible for protection following ischaemic preconditioning. Previous studies have demonstrated the requirement for G-protein coupled receptor activation, which stimulates phospholipase C via the activation of the G-protein α-subunits, $G_i/(G_q)$. One of the effects of phospholipase C activation is the production of diacylglycerol, which activates protein kinase C (PKC).

The PKC family is composed of at least 10 different isoforms. Non-isoform-selective PKC inhibitors abolish preconditioning, demonstrating the importance of this kinase family, however the particular isoform(s) responsible for protection is/are unknown. We used hearts from mice deficient in PKCε protein to study the role of this PKC isoform in preconditioning. Evidence is presented to suggest that PKCε is essential

for the reduction in infarct size seen following IP, but is not associated with the improvement in contractile recovery.

Mitogen-activated protein kinase (MAPK) activation has been implicated in protection downstream of PKC. Therefore, we examined the interplay between these kinases in a characterised model of ischaemic preconditioning in cultured rat neonatal ventricular cardiocytes. Of all the MAPK isoforms, only p38 was activated during simulated ischaemia in neonatal rat cardiac myocytes. Surprisingly, rather than enhancing, preconditioning/PKC activation diminished p38 activity during ischaemia. This inhibition of p38 contributes to protection, since pharmacological inhibition also protects against simulated ischaemia.

To assess the relative contributions of different p38 isoforms we used ectopic expression of p38α and p38β. Data is presented that suggests p38α is the isoform activated during ischaemia and inhibited by preconditioning, whereas p38β is inhibited during ischaemia. The detrimental effects of p38α activation are confirmed using a dominant negative p38α mutant, which protects myocytes against simulated ischaemia.

In conclusion, preconditioning protects myocytes by selective activation of PKCε. One of the downstream pathways that contribute to this protection is the inhibition of p38α phosphorylation during ischaemia (Figure 7-2).

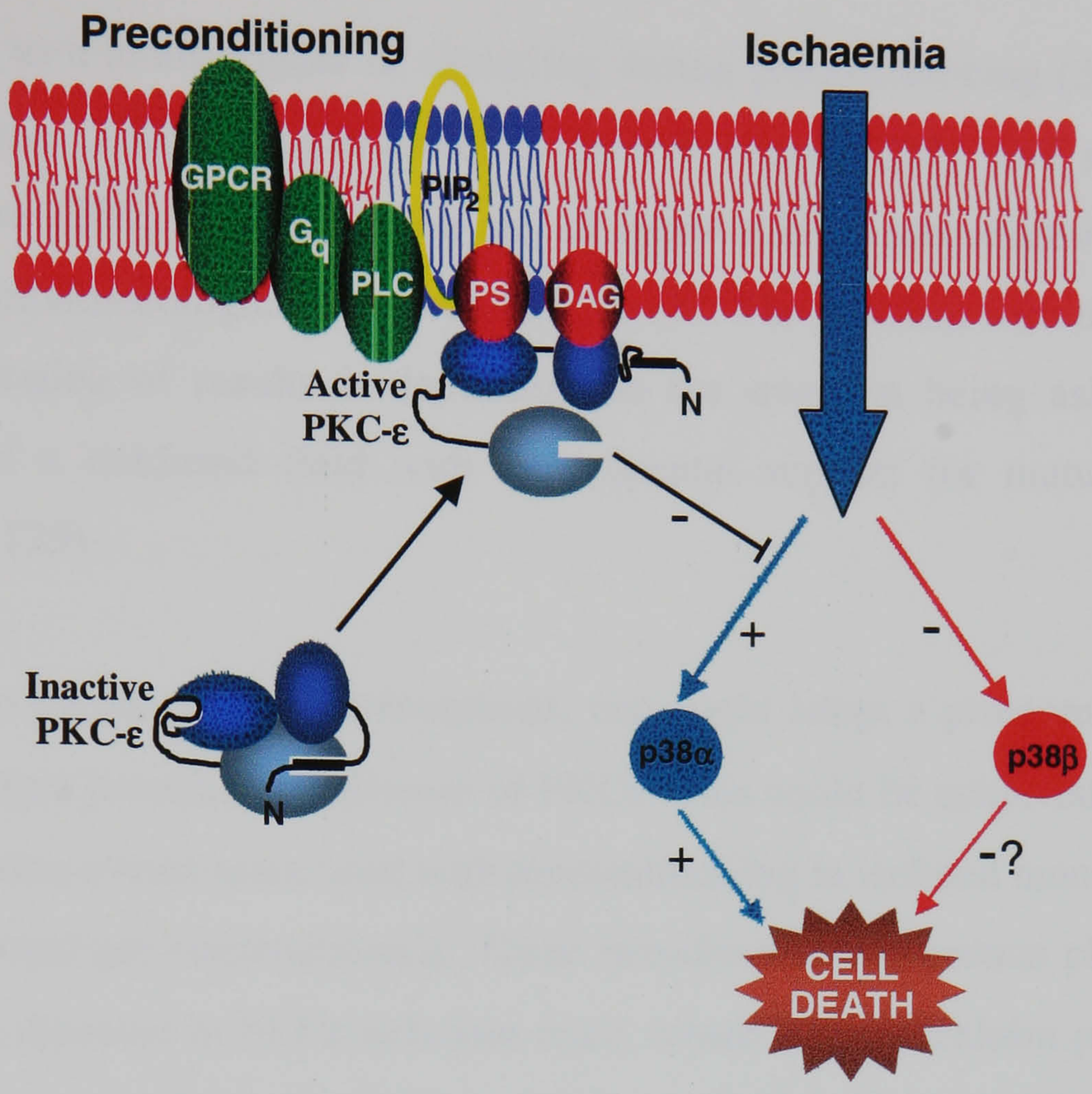

Figure 7-2: Summary of conclusions, based on the results presented within this thesis.

3 DESIGN OF FUTURE STUDIES

3.1 Protein kinase C

Since the discovery that PKC activation is essential for preconditioning, the search for its downstream targets have been exclusively hypothesis driven. These hypotheses are based on whether known effectors of preconditioning are PKC substrates. For example, the activation of the ATP-sensitive potassium (K_{ATP}) channel downstream of the adenosine receptor was originally reported as the "end-effector" of preconditioning (155). Since adenosine receptors couple to PKC via G-proteins, it was logical to assume that PKC activation may be necessary for the activation of K_{ATP} channels. Thus, perhaps not surprisingly, PKC activation was soon shown to be critical to K_{ATP} channel opening and protection (224, 225). However, this early interpretation may prove incorrect since the most recent evidence suggests this

channel acts as a redox trigger of signalling during preconditioning (369). Similarly, tyrosine kinase (130), mitogen-activated protein kinase (223), and stress-activated protein kinase (299, 370) activation are all implicated in preconditioning and have been linked pharmacologically to protein kinase C. The problem with this approach is that interpretation of results is dependent on the question being asked. The final picture is of a confused field with experimental support for mutually exclusive hypotheses (125).

In attempt to escape these preconceptions, one could adopt a proteomic approach to screen for target proteins downstream of PKCε. This could be achieved by examining phosphorylation events associated with preconditioning in isolated mouse hearts using two-dimensional gel electrophoresis. These two-dimensional protein phosphorylation maps can be repeated in PKCε deficient mice, which will not exhibit preconditioning despite exposure to identical patterns of ischaemia. By examining the differences in phosphorylation patterns in the presence and absence of PKCε it may be possible to identify new downstream candidate proteins. This approach can also be employed by comparing preconditioned and control hearts, but the lack of ischaemia in the controls would be expected to cause large changes in protein phosphorylation patterns that are unrelated to protection. The advantage of the knockout approach, is the only difference between groups, as well as the ability to precondition, is the presence of PKCε.

The findings presented in this thesis support the need to design experiments to examine MAPK activation following preconditioning in the PKCε deficient mice. If, as suggested in Chapter 1, the decrease in p38 activation is a result of PKC activation, which contributes to protection, then this decrease should be absent following preconditioning in PKCε knockout hearts.

Although PKCε is responsible for preconditioning in this model, it is unknown whether this is a unique ability of this isoform to protect, or whether preconditioning only activates this PKC isoform. To address this question, experiments should be

designed to test whether PKC activators can protect mouse hearts in the absence of PKCε.

A recent study has suggested that mitochondrial K_{ATP} channel opening can trigger the preconditioned state by the generation of free radicals, which presumably activate intracellular kinases (369). This has raised doubts as to whether mitochondrial K_{ATP} channel function as a trigger, or effector, of preconditioning (175). Experiments in the PKCε knockout mouse model could help to determine the position of K_{ATP} channels in relation to PKCε, by the administration of a K_{ATP} channel opener (diazoxide) in heterozygous and knockout mice.

3.2 Mitogen-activated protein kinases

At the present time, much controversy surrounds the role of p38-MAPK during both preconditioning and ischaemia. Most preconditioning investigators believe that activation of p38-MAPK contributes to protection. Thus, activation of PKC increases p38 activity, which causes MAPKAPK-2 phosphorylation and protection (presumably by phosphorylating a protective downstream substrate such as HSP27). During ischaemia however, most studies, including those described within this thesis, show a detrimental role for p38-MAPK activation. We believe a contributory factor to these disparities is the reliance on the p38-MAPK inhibitor, SB203580, as the key manipulation to differentiate benefit from harm. This inhibitor is non-specific and known to inhibit and activate kinases other than p38. Furthermore, the disregard to the possible opposing effects of p38 isoforms may also contribute to the confusion.

In attempt to overcome these uncertainties, future studies should be designed to delineate the cause and consequence of differential p38 isoform activation in the whole heart. To do this, the studies assessing isoform activation in neonatal cardiac myocytes can be repeated in the whole heart by immunoprecipitation of phosphorylated p38 during ischaemia followed by Western blotting with anti-p38α and p38β antibodies. To address whether MKK3 activation is responsible for p38α activation during ischaemia, we have recently obtained mice deficient in MKK3

protein. Preliminary studies to ascertain whether p38 activation during ischaemia is abolished in these mice are currently being undertaken. If p38 phosphorylation were inhibited, this would provide an ideal opportunity to assess the consequence of p38α activation during ischaemia in the whole heart.

In regard to preconditioning, we have acquired mice deficient in the downstream p38 substrate, MAPKAPK-2. If the protective p38 hypothesis is correct, then these mice should lack the ability to precondition. Studies are due to start that will investigate whether these mice precondition *in vivo*.

A final possibility that deserves investigation is whether transient p38 activation during preconditioning modulates the sustained p38 phosphorylation during ischaemia. This hypothesis could unify the findings that p38 is both good and bad, because transient p38 activation during preconditioning may protect by inhibiting the detrimental sustained activation during ischaemia. To address this hypothesis one could examine the activation of p38-MAPK during ischaemia following preconditioning in the presence or absence of p38 inhibitors during preconditioning.

4 IMPLICATIONS OF THE WORK DESCRIBED

The field of myocardial protection has been an area of intense research activity for the past 3 decades. The attraction lies in the fact that myocardial infarction is the major cause of death in the Western World, and that manoeuvres that slow or prevent ischaemic cell death are likely to have important clinical implications. Ischaemic preconditioning is second only to reperfusion as an efficient means of protecting the heart against ischaemia. The studies presented within this thesis highlight two related intracellular mechanisms involved in protection, namely PKCε activation and p38α inhibition. These findings may represent viable clinical targets, but should at least form the basis of future studies designed to dissect the signalling pathways initiated or inhibited by preconditioning, with the long-term aim of discovering the "end-effector" of protection.

APPENDICES

1 SOLUTIONS

General laboratory chemicals were of analytical grade, solid chemicals were dissolved in DI H_2O (resistivity >18mΩ/cm^3) unless otherwise stated, and adjusted to the required pH with either HCl or NaOH.

1.1 Tissue culture

1.1.1 Full growth media

Dulbecco's Modified Eagles Medium (DMEM)	500mls
Foetal Calf Serum	50mls
Penicillin/Streptomycin (100units/ml)	5mls

1.1.2 Serum free media

DMEM	500mls
Penicillin/Streptomycin (100units/ml)	5mls

1.1.3 Phosphate-buffered saline

This was made from pre-formed tablets (GibcoBRL, UK) consisting of:

NaCl	4.0g
KCl	0.1g
Na_2PO_4	0.72g
KH_2PO_4	0.12g

Dissolved in 500mls DI H_2O.

1.1.4 Trypsin solution

Trypsin	10mls
PBS	40mls

1.2 Neonatal myocyte isolation and culture

1.2.1 ADS buffer (10×)

This was used (after diluting 1:10 in DI H_2O) to buffer the neonatal cardiac myocytes during isolation. The stock solution consisted of 120mM NaCl, 18mM HEPES, 1mM NaH_2PO_4, 5mM Glucose, 5mM KCl, and 0.5mM $MgSO_4$.

NaCl	6.8g
HEPES	4.76g
NaH_2PO_4	0.12g
Glucose	1.0g
KCl	0.4g
$MgSO_4$	0.1g

The pH was adjusted to 7.35 with 1N NaOH and the solution brought up to 1 litre with DI H_2O. The solution was then filtered into a 1 litre autoclaved Duran, using a 0.22µg Nalgene bottle-top filter, and stored at 4°C.

1.2.2 Digestion buffer

10×ADS buffer (as above)	10mls
Collagenase Type-2 (317units/mg)	30mg
Pancreatin (0.125g/ml)	500µl

Made up to 100mls in sterile DI H_2O and bubbled with 100% O_2 for 60 minutes before filter sterilisation.

1.2.3 Plating media

This was used for primary cultures of neonatal myocytes during the first 24 hours following isolation.

DMEM	335mls
Medium 199 (with hanks' salts)	85mls
Horse serum	50mls
Foetal calf serum	25mls
Penicillin/Streptomycin (100units/ml)	5mls

1.2.4 Maintenance media

24 hours following isolation, neonatal myocytes were cultured in maintenance media, containing low serum, prior to experimentation.

DMEM	390mls
M 199	100mls
Foetal calf serum	5mls
Penicillin/Streptomycin (100units/ml)	5mls

1.3 Ischaemia studies in neonatal myocytes

1.3.1 Simulated ischaemia buffer

Ischaemia is simulated by exposure to 1ml of ischaemia buffer containing: 118mM NaCl, 24mM $NaHCO_3$, 1mM NaH_2PO_4, 2.5mM $CaCl_2$, 1.2mM $MgCl_2$, 0.5mM sodium·EDTA·$2H_2O$, 20mM sodium lactate, and 16mM KCl, pH 6.2.

NaCl (1.18M)	10mls
$NaHCO_3$ (240mM)	10mls

KCl (160mM)	10mls
NaH_2PO_4 (100mM)	1ml
$CaCl_2$ (250mM)	1ml
$MgCl_2$ (120mM)	1ml
sodium·EDTA·$2H_2O$ (50mM)	1ml
sodium lactate (60%, w/v)	175µl
DI H_2O	60mls

pH to 6.2 with 1M HCl and made up to 100mls in DI H_2O.

1.3.2 Cell viability assay

1.3.2.1 MTT solution

An MTT solution (5mg/ml, w/v) is used to assess cell viability, since it is reduced from a yellow to a blue dye in living cells.

MTT	50mg
PBS	10mls

1.3.2.2 Solubilisation solution

The MTT reaction is stopped by the addition of solubilisation solution containing 0.1M HCl and 10% (v/v) Triton X-100 in isopropranol.

HCl (1M)	10mls
Triton X-100	10mls
Isopropranol	80mls

1.4 Viral Production

1.4.1 Caesium chloride solutions

Caesium chloride is commonly used to separate DNA and adenovirus from cellular debris, RNA and empty (defective) capsids by ultracentrifugation on buoyant density gradients. For separation of recombinant adenovirus from defective virions three different buoyant density gradients were used:

1.25g/ml buoyant density CsCl	36.2g
1.35g/ml buoyant density CsCl	51.2g
1.40g/ml buoyant density CsCl	62g

100mls of PBS was added to the caesium chloride and stored at room temperature.
For separation of DNA from RNA and cellular protein a 65% (w/v) solution of caesium chloride was used.

CsCl	65g

Made up to 100mls with DI H_2O.

1.4.2 Dialysis buffer

This buffer was used to dilute residual caesium chloride from recombinant adenoviral preparations. It comprises of 10% (v/v) glycerol, 1mM $MgCl_2$, 10mM TRIS at pH 7.4.

Glycerol	400mls
1M $MgCl_2$ stock solution	4mls
1M TRIS (pH 7.4)	40mls

Made up to 4 litres with DI H_2O.

1.4.3 Virus storage medium

This solution was used for storing and diluting aliquots of recombinant adenovirus. Its composition is the same as the dialysis buffer above, except the solutions is filtered through a 0.45µm filter prior to use. Aliquots were then stored at –70°C.

1.5 DNA preparation

1.5.1 Solution 1 (GET)

Consists of 50mM glucose, 10mM EDTA and 25mM Tris pH 8.0.

Glucose	0.9g
0.1M EDTA	10mls
0.1M Tris.HCl (pH 8.0)	25mls

Made up to 100mls with DI H_2O.

1.5.2 Solution 2 (SSDS)

Consists of 0.2M NaOH and 1% (w/v) SDS.

1M NaOH	20mls
10% SDS	10mls

Made up to 100mls with DI H_2O.

1.5.3 Solution 3

Consists of 3M potassium acetate and 11.5% (v/v) glacial acetic acid.

5M K.acetate	60mls
Glacial acetic acid	11.5mls
DI H_2O	28.5mls

1.5.4 Luria-Bertani medium

Luria-Bertani (LB) medium was used for the small and large-scale growth of bacteria. Selection antibiotic (ampicillin) was added to the media prior to use, at either room temperature or approximately 40°C for LB-agar.

To 950mls DI H_2O add:	
Bacto-tryptone	10g
Bacto-yeast extract	5g
NaCl	10g

The pH was then adjusted to 7.0 with addition of NaOH, and the volume of solution brought to 1 litre with DI H_2O and sterilised by autoclaving. To make LB-agar for plating bacteria, 15g/l of agarose was simply added to the media before autoclaving.

1.6 Agarose gel solutions

Agarose gels were used to separate and visualise DNA. They were composed of:

Agarose	0.6g
TBE (5×)	12mls
DI H_2O	48mls
Ethidium bromide (10mg/ml)	3µl

The mixture was heated in a 900W microwave (Sanyo Electric Co. Ltd., UK) to dissolve the agarose and poured into gel casts containing well-forming combs. The gel was then left to set for 1 hour at room temperature prior to use.

TBE stock (×5) consisted of:

Tris	54g
Boric acid	28g
EDTA (0.5M, pH 8.0)	20ml

Made up to 1 litre with DI H_2O.

1.7 Protein harvesting solutions

1.7.1 Sample buffer (2×)

Unless otherwise indicated, protein was harvested in boiling two-times sample buffer containing 20% (v/v) glycerol, 6% (w/v) SDS and 120mM Tris, at pH 6.8.

Glycerol	20mls
SDS	6g
Tris	1.4g

Made up to 100mls with DI H_2O.

1.7.2 Cell fractionation lysis buffer

The buffer used while fractionating samples contained either digitonin (0.05%, w/v) for cytosolic fractions or triton X-100 (1%, v/v) for particulate fractions, added to a lysis buffer containing 50mM TrisHCl (pH 7.5), 5mM EGTA, 2mM EDTA, 100mM NaF, 20µM Leupeptin, 10µM E-64, 120µM Pepstatin A, 200µM PMSF, and 5mM DTT.

TrisHCl (pH 7.5, 1M)	500µl
EGTA (0.05M)	1ml
EDTA (0.5M)	40µl
NaF (1M)	1ml
Leupeptin (1mg/ml)	100µl
E-64 (10mM)	10µl
Pepstatin A (1.4mg/ml)	600µl
PMSF (100mM)	20µl
DTT (1M)	50µl

Made up to 10mls with DI H_2O.

1.8 SDS-PAGE gel solutions

1.8.1 Polyacrylamide gel solutions

1.8.1.1 Resolving gel

Contains 1.5M Tris, 0.4% (w/v) SDS to pH 8.8 with HCl.

SDS	2g
Tris	90.9g
Add DI H_2O to 500mls.	

1.8.1.2 Stacking gel

Contains 0.5M Tris, 0.4% (w/v) SDS to pH 6.8 with HCl.

SDS	2g
Tris	30.25g
Add DI H_2O to 500mls	

Polyacrylamide gels were made, depending on concentration, according to Table 7-1.

	Stack	Base (Running) Gel					
Acrylamide Concentration	5%	5%	7%	10%	12.5%	15%	18%
Solution Added:							
Acrylamide (30%)	2ml	6ml	8.4ml	12ml	15ml	18ml	22.5ml
Resolving gel	-	9ml	9ml	9ml	9ml	9ml	9ml
Stacking gel	3ml	-	-	-	-	-	-
DI H_2O	7ml	21ml	18.6ml	15ml	12ml	5.4ml	4.5ml
APS (10%)	100µl	338µl	225µl	180µl	147µl	113µl	103µl
TEMED	10µl	15µl	15µl	15µl	15µl	15µl	15µl

Table 7-1: Solutions used to make polyacrylamide gels for SDS-PAGE.

1.8.2 Coomassie blue solution

Protein can be visualised in polyacrylamide gels by exposure to coomassie blue solution followed by destaining to remove background staining.

Coomassie brilliant blue R250	0.25g
Methanol	45mls
H_2O	45mls
Glacial acetic acid	10mls

1.8.3 Destain solution

Methanol	400mls
Glacial acetic acid	70mls
H_2O	530mls

1.8.4 Gel running buffer (10×)

Proteins were electrophoresed in running buffer containing 25mM Tris, 0.5M Glycine, and 0.1% (w/v) SDS.

Tris	15.2g
Glycine	72.1g
SDS	5g

Made up to 500mls with DI H_2O.

Before use the above buffer was diluted 1:10 in DI H_2O.

1.8.5 Gel transfer buffer (10×)

Proteins were transferred electrophoretically from SDS-PAGE gels onto nitrocellulose membranes in buffer containing 25mM Tris, 0.5M Glycine, and 20% (v/v) methanol.

Tris	15.2g
Glycine	72.1g

Made up to 500mls with DI H_2O.

Prior to use, 200mls of the above solution is added to 400mls methanol and 1400mls DI H_2O.

1.8.6 Western blot washing solution

Unless otherwise indicated, this solution was used to wash nitrocellulose membranes, between incubation of antibodies, to prevent non-specific binding.

PBS	500mls
Tween-20	500µl
Dried skimmed milk powder	0.5g

1.9 Langendorff-perfusion

1.9.1 Anaesthetic solution

Mice were anaesthetised by an intraperitoneal injection of ketamine (150mg/kg), xylazine (24mg/kg), and heparin (100IU) (adapted from Marber *et al.* (26)).

This was achieved by injecting the following solution at volumes dependent on mouse weight, as indicated in Table 7-2.

Ketamine (100 mg/ml)	2mls
Xylazine (20 mg/ml)	1.6mls
PBS	1.4mls
Heparin	4mls

Mouse weight (g)	Anaesthetic (µl)	Mouse weight (g)	Anaesthetic (µl)
25	203	36	242
26	211	37	250
27	218	38	258
28	227	39	266
29	235	40	274
30	243	41	285
31	251	42	293
32	259	43	301
33	267	44	309
34	275	45	318
35	283	46	326

Table 7-2: Dose of anaesthetic administered to mice.

1.9.2 Krebs-Henseleit Buffer

Krebs-Henseleit (K-H) buffer consisted of 118.5mM NaCl, 25.0mM $NaHCO_3$, 4.75mM KCl, 1.18mM KH_2PO_4, 1.19mM $MgSO_4$, 11.0mM d-glucose, and 1.41mM $CaCl_2$.

NaCl	34.63g
$NaHCO_3$	10.5g
KCl	1.77g
$MgSO_4$	1.47g
KH_2PO_4	0.8g
Glucose	9.9g
DI H_2O	4.5 litres

The solution is bubbled with 95%O_2/5%CO_2 for 10 minutes to lower the pH. Then 1.04g $CaCl_2$ was added and the solution brought up to 5 litres. The buffer was filtered through a 0.8μM cellulose nitrate micro-filter (Whatman, Maidstone, UK), and stored at 4°C for no longer than 1 day.

1.9.3 Triphenyl tetrazolium chloride staining solution

A 1% (w/v) triphenyl tetrazolium chloride (TTC) was used to demarcate the infarct zone in isolated mouse hearts.

Na_2HPO4 (8% solution, w/v)	0.8mls
NaH_2PO_4 (20% solution, w/v)	0.2mls
TTC	0.1g
DI H_2O	9mls

1.9.4 Mouse heart fixative

Following sectioning, the heart slices were fixed for 24 hours, prior to visualisation in a 10% (v/v) formaldehyde solution.

Formaldehyde (40%, v/v)	12.5mls
DI H_2O	37.5mls

2 TABLE OF ANTIBODIES

Antibody	Dilution	Type	Source
PKC-delta	1:1000	Mouse mAb	Transduction Labs
PKC-epsilon	1:1000	Mouse mAb	Transduction Labs
PKC-alpha	1:1000	Mouse mAb	Transduction Labs
ERK2	1:1000	Mouse mAb	Santa Cruz Biotech.
Phospho$^{Thr202/Tyr204}$-ERK1/2	1:1000	Rabbit pAb	Cell Signalling
p38	1:1000	Rabbit pAb	Cell Signalling
Phospho $^{Thr180/Tyr182}$-p38	1:1000	Rabbit pAb	Cell Signalling
Immobilised-phospho$^{Thr180/Tyr182}$-p38	1:50	Mouse mAb	Cell Signalling
Phospho$^{Thr183/Tyr185}$-JNK1/2	1:1000	Mouse mAb	Cell Signalling
Phospho^{Ser82}-HSP27	1:40	Mouse mAb	Dr RA Quinlan
Phospho^{Ser473}-PKB	1:1000	Rabbit pAb	Cell Signalling
Phospho^{Tyr308}-PKB	1:1000	Rabbit pAb	Cell Signalling
MKP-1	1:1000	Rabbit pAb	Santa Cruz Biotech.
p38-alpha	1:500	Rabbit pAb	Santa Cruz Biotech.
p38-beta	1:5000	Goat pAb	Santa Cruz Biotech.
Phospho-MKK3/6	1:1000	Mouse mAb	Santa Cruz Biotech.

Table 7-3: Description of antibodies used throughout this thesis.
Polyclonal (pAb) or monoclonal (mAb) antibodies were used at different dilutions as indicated. For phosphorylation-specific antibodies, the phosphorylation site(s) recognized by the respective antibodies are indicated in superscript.

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PUBLICATIONS

The pear-reviewed publications arising from work contained within this thesis are listed below:

Abstracts

American Heart Association 71st Scientific Session, Nov 1998.

Saurin et al. Negative regulation of p38 and p42/44 MAPK by PKC in a surrogate model of ischaemic preconditioning. *Circulation.* 98(17), 364 Suppl.

British Cardiac Society, May 1999.

Saurin et al. Inhibition, rather than activation, of p38 accounts for the cardioprotective effect of ischaemic preconditioning. *Heart.* May 1999.

European Society for Cardiology, August 1999.

Saurin et al. Inhibition of p38-alpha during index ischaemia contributes to the protection afforded by ischaemic preconditioning. *European Heart Journal.* August 1999.

American Heart Association, 72nd Scientific sessions, Nov 1999.

Saurin et al. Inhibition of p38-alpha may underlay protection in a surrogate model of ischaemic preconditioning. *Circulation.* 100(18). 2593. Suppl.

International society for heart research, American section, June 2000.

Martin et al. Ischaemic protection of cardiac myocytes expressing dominant negative p38-alpha. *J.Mol.Cell.Cardiol.* May 2000.

American Heart Association, 73rd Scientific sessions, Nov 2000.

Saurin et al. Ischaemic preconditioning in isolated mouse hearts – an essential role for PKC-ε. *Circulation.* Nov 2000.

Manuscripts

Published:

Saurin, AT., Martin, JL., Heads, RJ., Foley, C., Mockridge, JW., Wright, MJ., Wang, Y., and Marber, MS. (2000) The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *FASEB J.* **14,** 2237-2246.

Edwards, RJ., Saurin, AT., Rakhit, RH., and Marber, MS. (2000) Therapeutic potential of ischaemic preconditioning. *Br.J.Clin.Pharmacol.* **50,** 87.97.

Submitted:

Saurin, AT, Pennington, DJ, Raat NJH, Owen MJ, and Marber MS. Targeted disruption of the protein kinase C-epsilon gene abolishes the infarct size reduction that follows ischemic preconditioning of isolated buffer-perfused mouse hearts. *Submitted to J.Clin.Invest. (Dec 2000).*

The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes

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ABSTRACT **Activation of protein kinase C (PKC) and more recently mitogen-activated protein kinases (MAPKs) have been associated with the cardioprotective effect of ischemic preconditioning. We examined the interplay between these kinases in a characterized model of ischemic preconditioning in cultured rat neonatal ventricular cardiocytes where ectopic expression of active PKC-δ results in protection. Two members of the MAPK family, p38 and p42/44, were activated transiently during preconditioning by brief simulated ischemia/reoxygenation. Overexpression of active PKC-δ, rather than augmenting, completely abolished this activation. We therefore determined whether a similar process occurred during lethal prolonged simulated ischemia. In contrast to ischemia, brief, lethal-simulated ischemia activated only p38 (2.8±0.45 vs. basal, $P<0.01$), which was attenuated by expression of active PKC-δ or by preconditioning (0.48±0.1 vs. ischemia, $P<0.01$). To determine whether reduced p38 activation was the cause or an effect of protection, we used SB203580, a p38 inhibitor. SB203580 reduced ischemic injury (CK release 38.0±3.1%, LDH release 77.3±4.0%, and MTT bioreduction 127.1±4.8% of control, $n=20$, $P<0.05$). To determine whether p38 activation was isoform selective, myocytes were infected with adenoviruses encoding wild-type p38α or p38β. Transfected p38α and β show differential activation ($P<0.001$) during sustained simulated ischemia, with p38α remaining activated (1.48±0.36 vs. basal) but p38β deactivated (0.36±0.1 vs. basal, $P<0.01$). Prior preconditioning prevented the activation of p38α (0.65±0.11 vs. ischemia, $P<0.05$). Moreover, cells expressing a dominant negative p38α, which prevented ischemic p38 activation, were resistant to lethal simulated ischemia (CK release 82.9±3.9% and MTT bioreduction 130.2±6.5% of control, $n=8$, $P<0.05$). Thus, inhibition of p38α activation during ischemia reduces injury and may contribute to preconditioning-induced cardioprotection in this model.—Saurin, A. T., Martin, J. L., Heads, R. J., Foley, C., Mockridge, J. W., Wright, M. J., Wang, Y., Marber, M. S. The role of differential activation of p38-mitogen-activated protein kinase in preconditioned ventricular myocytes. *FASEB J.* 14, 2237–2246 (2000)**

Key Words: myocardial ischemia · cardioprotection · ischemic preconditioning · cytoprotection

THE TERM ISCHEMIC preconditioning was first coined by Murry and colleagues to describe a phenomenon where brief periods of sublethal ischemia protected or 'preconditioned' the heart against infarction caused by a subsequent more prolonged period of coronary artery occlusion (1). Since this initial discovery, preconditioning has become recognized as the most powerful form of cardioprotection other than reperfusion (2). Unfortunately, the protection afforded by a brief period of ischemia is short-lived (3) and cannot be renewed (4). In an attempt to overcome these deficiencies, the signaling pathways responsible for triggering and maintaining this powerful form of cardioprotection have become the focus of many investigators.

Early pharmacological studies delineated the mediator(s) responsible for adaptation in response to brief ischemia. These investigations in whole heart, and later in cell-based models (5), showed that protection was dependent on the activation of a wide variety of heptahelical transmembrane receptors including adenosine type 1 and 3 (6, 7), bradykinin (8), α_1-adrenergic (9), endothelin (10), angiotensin II (11), and delta-1 opioid receptors (12, 13). Although structurally diverse, these receptors have a common feature of coupling to protein kinase C (PKC) via GTP binding proteins. The importance of protein kinase C to ischemic preconditioning has been shown in a variety of studies in whole heart and

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in isolated ventricular cardiocytes (8, 11, 14–16). Although it is widely accepted that PKC plays a pivotal role in ischemic preconditioning, the relevant downstream signaling molecules remain a topic of intense investigation and controversy.

Apart from PKC, the only other well-accepted component of the preconditioning signaling pathway is the ATP-dependent potassium channel. It is uncertain, however, whether this channel lies upstream or downstream of PKC (17–19). Similarly, in preconditioned myocardium an increase in the activation of p38 and p42/44 mitogen-activated protein kinases (MAPK) has been linked to protection (20, 21). However, this is not consistent with the reduction in ischemic injury that accompanies p38-MAPK inhibition in similar models (22–24). These inconsistencies only serve to further fuel the investigation of signals distal to PKC.

Neonatal rat ventricular cardiac myocytes have been used as an archetypal model to interrogate the signal transduction cascades underlying cardiac hypertrophy (25). Hypertrophy and preconditioning have features in common, including agonists, which trigger (endothelin, norepinephrine, angiotensin II), and PKCs, which mediate the final response (25). These similarities have caused others (26–28) and us (29) to characterize models of ischemic preconditioning in this cell type (5). These and other cell-based models (13, 30–37) have the advantages of the *in vivo* features of ischemic preconditioning without the disadvantages of multiple cell types, low transfection efficiency and spatial heterogeneity in depth of ischemia and quality of reperfusion (5). Using such a model, we previously demonstrated that preconditioning is PKC dependent and can be mimicked by expression of constitutively active PKC-δ (29). Our aim was to use this characterized model to investigate the interplay between preconditioning, PKC, and MAPKs.

MATERIALS AND METHODS

Isolation and culture of rat ventricular cardiomyocytes

Neonatal rat ventricular myocytes were prepared from 1- to 2-day-old Sprague-Dawley rats as described previously (29, 38). Briefly, cells from neonatal rat ventricles were dispersed in a series of incubations at 37°C in HEPES-buffered salt solution containing 0.5 mg/ml collagenase and 0.6 mg/ml pancreatin. Dispersed cells were then preplated for at least 30 min to minimize fibroblast contamination, and the unattached cells were replated on 6-well gelatin-coated plates at a density of ~1 million cells/well. Fibroblast contamination was less than 5%. The cardiac myocytes were cultured in 4:1, Dulbecco's modified Eagle's medium: M199, supplemented with 10% horse serum, 5% fetal calf serum (FCS), and 100 units/ml penicillin/streptomycin at 37°C in room air with 5% CO_2 for the first 24 h. Thereafter, cells were maintained in an identical medium with a reduced serum concentration of 1% FCS. Under these conditions, in excess of 80% of cells beat spontaneously for up to 1 wk in culture. Experiments were performed after 2–4 days in culture.

cDNA constructs

The high efficiency eukaryotic expression plasmid pCAGGS was used for all PKC transfections (39). This plasmid contains the cytomegalovirus immediate early enhancer and chicken β-actin promoter with the first intron upstream of a multiple cloning site. It has been shown previously that this heterologous promoter is transcriptionally active in cardiac myocytes (40). PKC mutants were constructed as described previously (29). Two PKC isotypes were studied: *1)* wild-type PKC-δ; *2)* PKC-δ with a limited deletion of the inhibitory pseudosubstrate subdomain (residues 151–160). This mutant PKC isotype has been shown to code for a constitutively active functional protein (41, 42). All plasmids were purified by alkaline lysis of the bacterial host (DH5α), followed by polyethylene glycol precipitation.

Recombinant adenovirus vectors

Recombinant adenoviruses encoding wild-type p38α, wild-type p38β, or dominant negative p38α driven by a cytomegalovirus promoter were generated as described previously (43, 44). The dominant negative p38α has a mutated phosphorylation site ($TGY^{180-182}$ to AGF), rendering it resistant to phosphorylation (45). Recombinant adenoviruses were tested for transgene expression in cardiac myocytes by reverse transcriptase-polymerase chain reaction, Western blot, or kinase assays. The concentrated recombinant adenoviruses were prepared and titered as described (44).

Transfection of neonatal cardiomyocytes

Cardiocytes at 70–80% confluency were transfected with pCAGGS expression plasmid by an integrin targeting peptide-mediated transfection procedure described previously (46). The peptide–Lipofectin complexes were prepared by mixing 40 μl peptide (0.1% w/v) and 0.75 μl Lipofectin (Life Technologies Ltd., Paisley, U.K.). DNA (0.01% w/v) in optimem was added to peptide–Lipofectin complex at a ratio of 2.5:1 (v/v). DNA–peptide–Lipofectin complexes were allowed to stand for 1 h at room temperature before use; 100 μl of this mix was diluted to 1 ml in optimem and added to one well of a 6-well plate. Cells were then incubated overnight at 37°C in room air supplemented with 5% CO_2. Thereafter, complex/optimem was removed and replaced with maintenance medium containing 1% FCS and the cells were returned to the incubator. Cell extracts were assayed for protein 48–72 h post-transfection. By using pCAGGS-GFP as a reporter, transfection efficiency was consistently between 20 and 30%.

Cells maintained in serum-free medium were infected with adenoviruses at a multiplicity of infection of 10 plaque-forming units/cell for 1 h at 37°C in room air containing 5% CO_2. Cells were then cultured in maintenance medium containing 1% FCS for an additional 48–72 h before biochemical analysis.

Ischemia model

The cells were washed once with phosphate-buffered saline (PBS) before addition of 1 ml of ischemia buffer (118 mM NaCl, 24 mM $NaHCO_3$, 1 mM NaH_2PO_4, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 0.5 mM sodium·EDTA·$2H_2O$, 20 mM sodium

lactate, and 16 mM KCl, pH 6.2) pregassed with 5% CO_2, 95% argon. On addition of ischemia buffer, spontaneous contraction within the monolayer ceased. Cells were then transferred to anaerobic GasPak pouches (Becton Dickinson, Sparks, Md.) and incubated at 37°C for up to 6 h. The O_2 content of the atmosphere inside the pouches was <1% for the duration of the experiment as measured by an anaerobic indicator.

Measurement of enzyme release

Upon opening the ischemia chamber (reoxygenation), 200 μl samples of the ischemia buffer were gently collected for the determination of creatine kinase (CK) and lactate dehydrogenase (LDH). The next day a spectrophotometric CK and LDH enzyme assay was performed with Boehringer Mannheim (Mannheim, Germany; MPR-1) and Sigma (St. Louis, Mo.; TOX-7) assay kits, respectively.

Evaluation of cell viability

After simulated ischemia, cells were reoxygenated in maintenance medium containing 1% FCS. After 2 h, medium was aspirated and cells incubated in 500 μl PBS containing 2.5 mg 3-(4,5-dimethylthiaziazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) for 30 min at 37°C in room air containing 5% CO_2. During this incubation the tetrazolium component of the dye is reduced, in metabolically active cells, to a formazan dye. Thereafter the reaction was terminated by addition of 500 μl solubilization solution (0.1 mol/l HCl, 10% triton X100, in isopropanol) and the absorbance of the lysate was recorded at 570 nm using an ELISA reader.

Western blot analysis

Cells from parallel plates were washed three times in ice-cold PBS and harvested in 1 ml of hot electrophoresis sample buffer (250 mM Tris-HCl, 4% sodium dodecyl sulfate, 10% glycerol, and 2% β-mercaptoethanol, pH 6.8), then boiled for an additional 5 min. The cell extracts were then centrifuged for 5 min to remove insoluble material; 0.003% bromphenol blue was added and the samples were loaded on a 10% polyacrylamide gel. After 1-dimensional separation the protein was electrophoretically transferred to nitrocellulose membranes (Hybond C, Amersham, U.K.). Coomassie staining of identically loaded gels confirmed uniform protein loading.

Blots were sequentially probed with either murine monoclonal antibodies specific for ERK2 (Santa Cruz Biotechnology, Santa Cruz, Calif.) and a peroxidase-conjugated rabbit anti-mouse IgG secondary antibody (DAKO A/S, Glostrup, Denmark) or rabbit polyclonal antibodies specific for p38, phospho-p38, or phospho-p42/44 (New England BioLabs, Hitchin, U.K.) and a peroxidase-conjugated swine anti-rabbit IgG secondary antibody (DAKO A/S). Secondary antibodies were then detected by incubation of the nitrocellulose with enhanced chemiluminescence (ECL, Amersham, Little Chalfont, U.K.) for 60 s prior to exposure to autoradiography film. The densities of all Western blot bands were analyzed using NIH Image version 1.61.

Statistical analysis

All values are expressed as mean ±SE. Data for individual treatments were collected from no more than two wells from each experimental preparation. The 'n' numbers under 'results' relate to the number of wells from which data were obtained. For each treatment, mean values were pooled to allow statistical comparisons. Statistical comparisons between groups were performed by one-way analysis of variance. All *post hoc* comparisons were by the Fischer protected least significant difference method. All analyses were performed using Statview version 4.0 statistical package (Abacus Concepts Inc., Berkeley, Calif.). A probability value ≤ 0.05 was considered significant.

RESULTS

Activation of MAPKs during preconditioning in isolated neonatal cardiac myocytes

Preconditioning with 90 min ischemia and 30 min reoxygenation has been shown to delay cell death in response to subsequent prolonged ischemia in our model (29). p38-MAPK activation has been reported during preconditioning-like ischemia in the intact heart (47). It has been proposed that this activation is associated with subsequent protection against lethal ischemia (20). Therefore, we wished to investigate whether similar activation occurred during preconditioning with simulated ischemia in our model. Cardiocytes were harvested at various time points during an acute preconditioning stimulus of 90 min ischemia and 30 min reoxygenation. Cell lysates were probed with phospho-specific antibodies for p38, p42/44, or p46/54 to quantify MAPK activation. These preliminary experiments demonstrated that although no p46/54 phosphorylation could be detected, p38 and p42/44 were transiently activated during reoxygenation after sublethal ischemia (results not shown) and that at 10 min reoxygenation both p38 and p42/44 displayed maximal activation (see **Fig. 1**).

The specific phosphorylation of MAPKs during reoxygenation may be a consequence of PKC activation, since it had been reported previously that PKC is activated at the onset of reperfusion (48). If this is true in our model then we should observe a comparable level of MAPK phosphorylation in cells overexpressing active PKC-δ even in the absence of preconditioning ischemia. Therefore, to test this hypothesis we transfected myocytes with constitutively active PKC-δ in an attempt to mimic the pattern of p38 and p42/44 phosphorylation seen after 90 min simulated ischemia and 10 min reoxygenation.

Effect of PKC-δ on MAPK activation in cardiac myocytes

To determine whether PKC-δ could activate MAPKs, myocytes were transfected with the eukaryotic expression plasmid pCAGGS encoding either wild-type PKC-δ, active PKC-δ, or vector alone. After 48–72 h, myocytes were either harvested, to assess basal MAPK phosphorylation, or subjected to 90 min ischemia and 10 min reoxygenation to examine precondition-

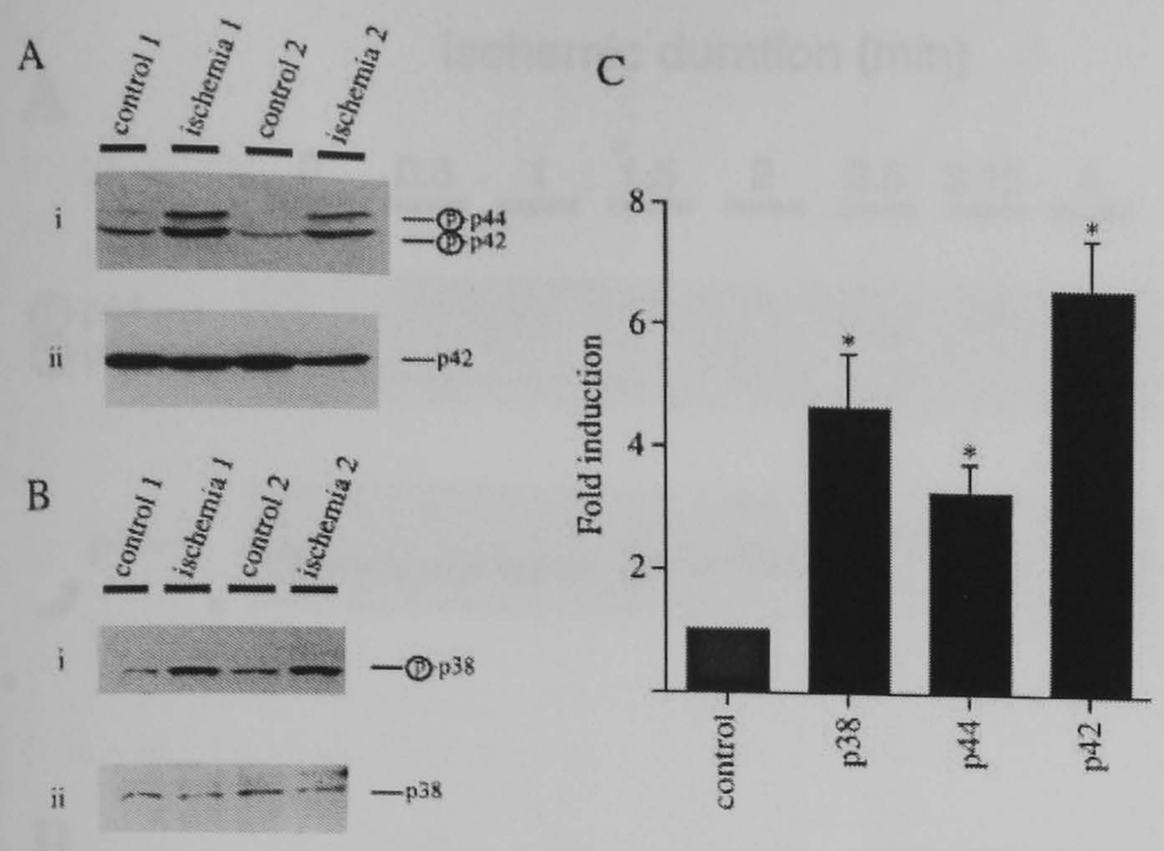

Figure 1. Activation of p38 and p42/44 MAPK during reoxygenation after 90 min simulated ischemia. Myocyte cell lysates were prepared from naive cells (control) and from wells after 90 min SI and 10 min reoxygenation (ischemia). Each pair of samples is from a different cardiocyte preparation exposed independently to simulated ischemia and reoxygenation. Samples are probed with dual phospho-specific MAPK antibodies for p42/44 (*A*i) and p38 (*B*i) or antibodies detecting total p42 (*A*ii) and total p38 (*B*ii). Panel *C* represents the mean activation of p38, p42 and p44 during simulated ischemia/reoxygenation expressed as a ratio of that seen in naive cells in 5 independent experiments. *$P<0.01$ vs. naive control, $n=5$.

ing-induced MAPK activation. It is apparent from **Fig. 2** that overexpression of active PKC-δ has no effect on basal p42/44 phosphorylation compared to controls (Fig. 2*A*i). Surprisingly, however, the increase in p42/44 phosphorylation observed during preconditioning was completely abolished in the presence of active PKC-δ; this is not due to a down-regulation of MAPK, as the total amounts of p42 remain constant between treatments (Fig. 2 *A*ii). In contrast, p38 exhibits a higher basal activation in cells expressing active PKC-δ; however, once again the preconditioning-induced increase in p38 phosphorylation is completely abolished (see Fig. 2*B*i).

These data did not support our original hypothesis that PKC-δ protects by causing the same activation of MAPKs that occurs with preconditioning. In contrast and paradoxically, these data suggest that PKC-δ inhibits MAPK activation in response to ischemia/reoxygenation. It is possible that this unexpected negative regulation may be the mechanism through with PKC-δ protects against lethal ischemia (29). This alternative hypothesis is consistent with reports examining the role of p38 during ischemia in the absence of preconditioning, which show that inhibition is protective (22–24). However, for PKC-δ to protect via this mechanism, the same inhibitory effect on MAPKs must occur during prolonged lethal ischemia. Therefore, we delineated the MAPK pathways that were activated by simulated ischemia alone.

Activation of MAPKs during prolonged simulated ischemia

To quantify the level of MAPK activation during ischemia, cardiocytes were subjected to varying duration's of simulated ischemia before cells were harvested and constituent proteins probed with anti-phospho-p38 and -p42/44 antibodies (see **Fig. 3**). In agreement with other recent reports (22, 49), we found that during ischemia p38 exhibits a prolonged activation, which is maximal after 2.5 h ischemia (Fig. 3*B*). In contrast, phospho-p42/44 MAPK is inhibited below basal levels for the entire duration of ischemia (Fig. 3*A*). This suppression of p42/44 during ischemia eliminates the possibility that PKC-δ protects through negative regulation of p42/44 phosphorylation. Therefore, we wished to examine

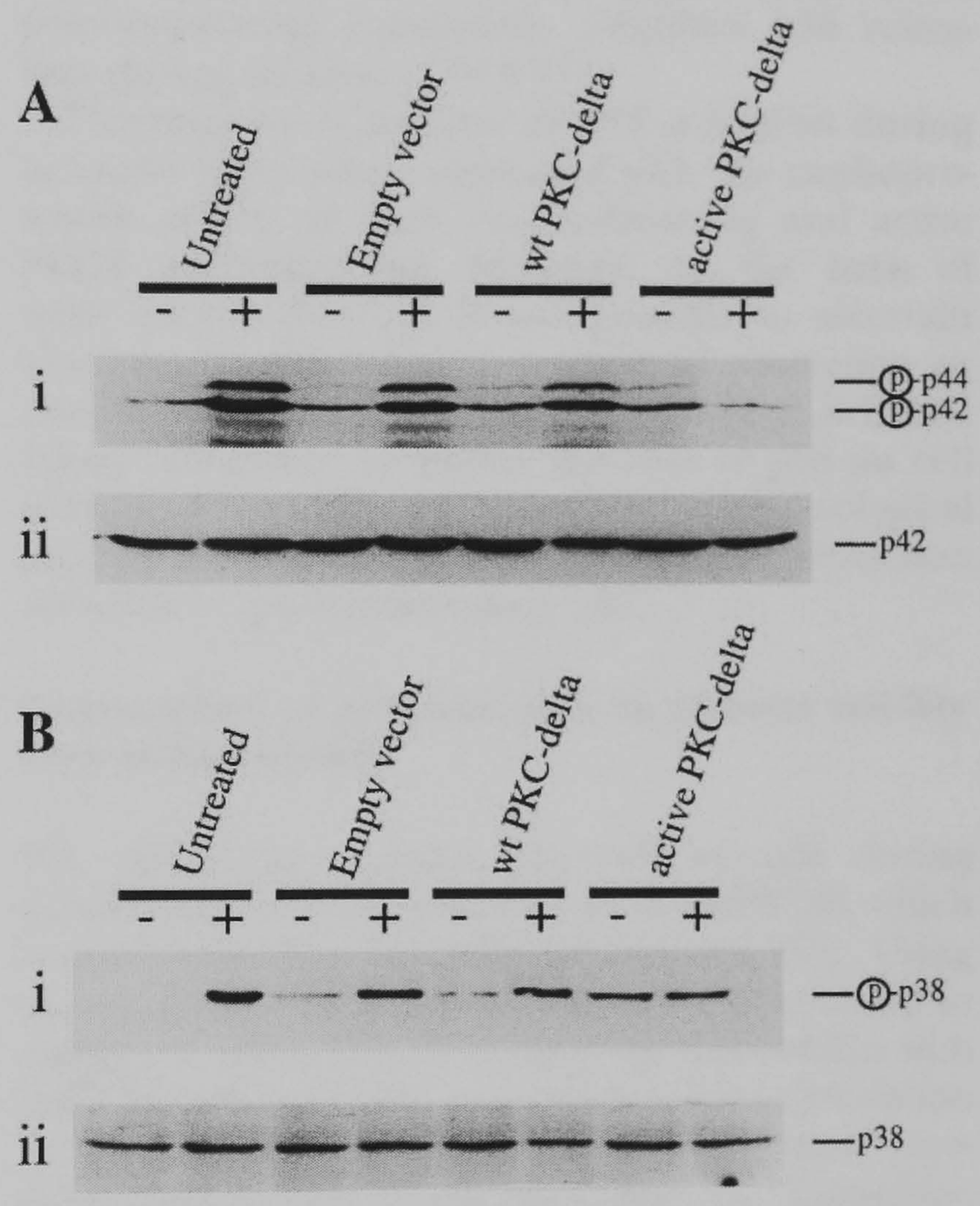

Figure 2. Activation of p38 and p42/44 MAPK after preconditioning in myocytes overexpressing PKC-δ. *A)* Western blot probed with anti-phospho-p42/44 (i) or anti-p42 (ii). *B)* Western blot probed with anti-phospho-p38 (i) or anti-p38 (ii). Constituent proteins in both panels were derived from the same experimental groups. Naive cells (−) were compared to preconditioned (+) in untreated cardiocytes, cells transfected with expression plasmid containing an empty multiple cloning site, encoding wild-type PKC-δ, or constitutively active PKC-δ (see Materials and Methods). *A)* Maximal p42/44 MAPK activation was assessed in cell lysates harvested after 10 min reoxygenation after 90 min simulated ischemia, using dual phospho-specific p42/44 antibodies (i). Total p42 levels were detected using a monoclonal anti-p42 antibody (ii). *B)* p38 activation was detected using phospho specific p38 antibodies (i) and total p38 was detected using anti-p38 antibodies (ii).

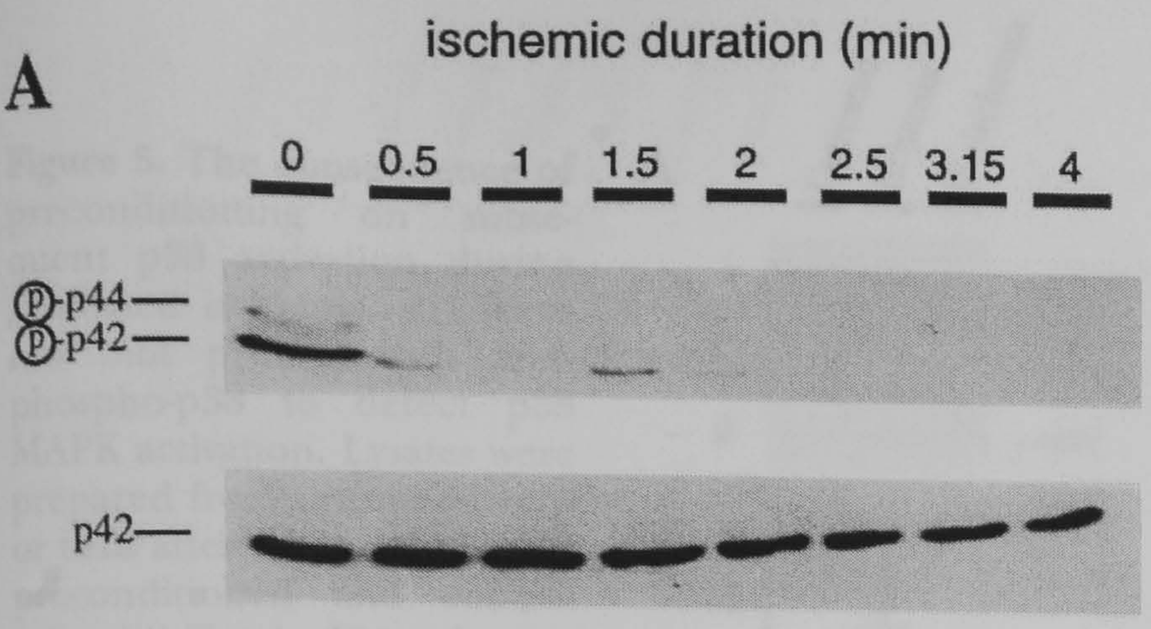

Figure 3. Time course of p38 and p42/44 MAPK activation during prolonged simulated ischemia in naive cardiocytes. Cardiocytes were subjected to varying duration's of simulated ischemia (0–6 h) before cells were lysed for Western blot analysis. *A)* Constituent proteins were probed with dualphospho-specific p42/44 antibodies to assess p42/44 MAPK activation or anti-p42 antibodies to examine p42 levels. *B)* Samples identical to panel *A* were probed with anti-dualphospho-p38 antibodies to detect p38 activation or anti-p38 antibodies to assess total p38 levels.

the result of PKC-δ overexpression on the activation of p38 during simulated ischemia.

Effect of active PKC-δ on p38 phosphorylation during lethal simulated ischemia

Isolated myocytes were either cultured under normal conditions (untreated) or transfected with plasmids encoding wild-type or active PKC-δ; 48–72 h post-transfection, cardiocytes were subjected to 2.5 h simulated ischemia and immediately harvested for Western blot analysis. Lysates were probed with anti-p38 or anti-phospho-p38 antibodies to detect the effect of PKC-δ overexpression on either p38 induction or activation (see **Fig. 4**). Overexpression of wild-type PKC-δ increased p38 phosphorylation during ischemia (Fig. 4i), although comparable results with empty vector alone suggest this is not a consequence of PKC-δ (results not shown). However, transfection of active PKC-δ significantly attenuated the ischemia-induced p38 activation (Fig. 4i), although there was no effect on total p38 levels (Fig. 4ii).

We have shown previously that specific activation of PKC-δ during ischemia protects myocytes against cell death (29) and, as we show here, PKC-δ activation causes an inhibition of p38 phosphorylation. Since preconditioning also protects by activating PKC, we sought to compare its effect on p38 activation by examining ischemia-induced phosphorylation in naive and preconditioned cells.

Activation of p38 during simulated ischemia after preconditioning

Cardiocytes were preconditioned with 90 min simulated ischemia and 30 min reoxygenation. These and untreated myocytes were then subjected to 2.5 h ischemia to maximally activate p38. Thereafter p38 phosphorylation was assessed, as before, by immunoblotting with phospho-p38 antibodies. **Figure 5** shows that, akin to active PKC-δ overexpression, preconditioning consistently inhibited p38 activation during ischemia ($P<0.01$).

The negative regulation of p38 activation during ischemia is therefore associated with the cardioprotective effects of both preconditioning and active PKC-δ overexpression. However, on the basis of these results alone, it is not possible to ascertain whether the inhibition is a cause of protection or simply a consequence of the attenuation of ischemic injury. Therefore, to define the role of p38 on cell viability after ischemia, we used pharmacological inhibition of p38 in an attempt to mimic protection afforded by preconditioning/PKC.

Consequence of p38 activation on myocyte viability after lethal ischemia

We wished to examine the role of p38 during ischemia by inhibiting activity with SB203580, which reversibly binds to the ATP binding site (50). Using two separate end points of CK and LDH release to assess cell injury and by measuring cell viability with MTT bioreduction, we can clearly see that inhibition of p38 during ischemia significantly protects myocytes against cell death (see **Fig. 6**). Therefore, during ischemia p38 activation is detrimental to myocyte survival, because specific inhibition of this

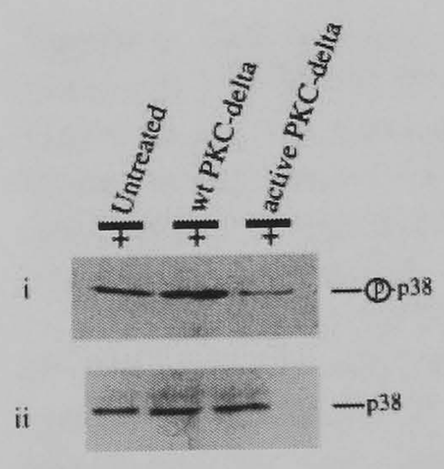

Figure 4. Effect of active PKC-δ expression on p38 phosphorylation during ischemia. 48–72 h post-transfection, myocytes were subjected to 2.5 h simulated ischemia and harvested for Western blot analysis to detect p38 dual phosphorylation. Using dual phospho-specific p38 antibodies, ischemia-induced p38 activation was detected in untreated cells or cells transfected with wild-type or active PKC-δ (i). Total p38 levels in the same cells were assessed with anti-p38 (ii).

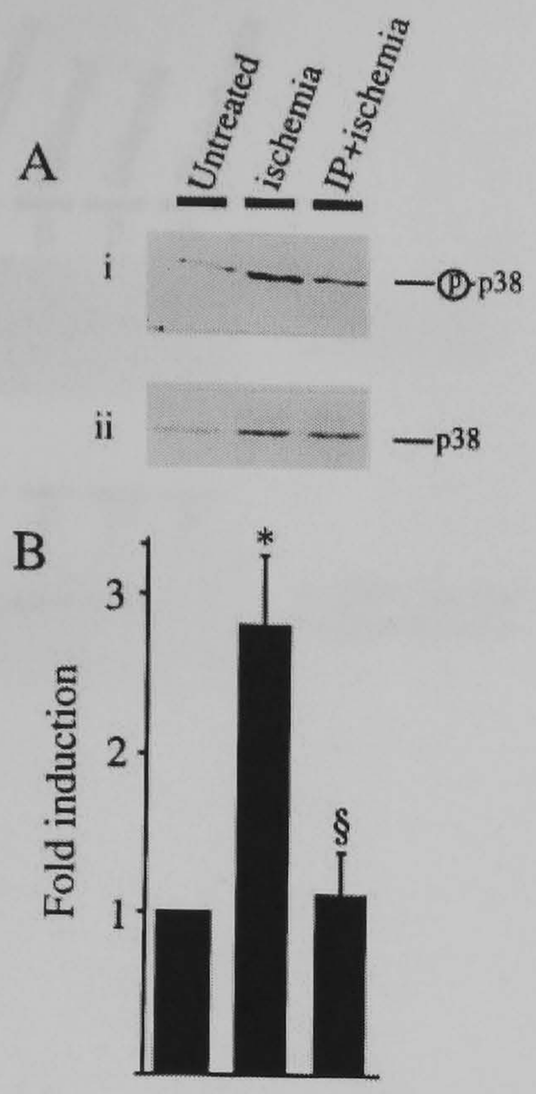

Figure 5. The consequence of preconditioning on subsequent p38 activation during simulated ischemia. *Ai)* Western blot probed with anti-phospho-p38 to detect p38 MAPK activation. Lysates were prepared from untreated cells or cells after 2.5 h ischemia in preconditioned and control cells. *Aii*) Total p38 levels were detected in the same cells with anti-p38. *B)* Mean p38 activation compared to untreated controls (n=4). *P<0.01, p38 activation during ischemia vs. nonischemic/untreated. §P<0.01, p38 activation during ischemia with vs. without preconditioning.

pathway is sufficient to protect myocytes. Hence, the attenuation of p38-MAPK activation by PKC-δ overexpression and preconditioning contributes to the protective effect of both these treatments.

The measurement of total p38 activation through the use of dual phospho-specific antibodies has limitations. In light of recent reports characterizing new p38 isoforms that share the same TGY motif recognized by commercially available antibodies, it has become apparent that isotypes may differ significantly in function, if not in structure. In fact, Wang and co-workers have proposed opposing roles for p38α and p38β on myocyte survival, postulating that p38α may be responsible for cell death and apoptosis, and p38β for hypertrophy and survival (51). Therefore, an increase in p38α activation with a comparable decrease in p38β phosphorylation may not alter total p38 activation as detected by phospho-specific antibodies. But if the Wang hypothesis were correct, we would expect such a change in the balance of active p38 isoforms to cause a large decrease in cell viability. Thus, there is a possibility that this decrease in viability may not be correctly attributed to p38 activation using currently available antibodies, so we wanted to examine the isotype specific activation of p38 during ischemia in our model.

Activation of p38 isotypes during simulated ischemia

To look at the activation of individual isotypes, we used recombinant adenoviruses as an efficient gene delivery vector to express various p38 signaling molecules (51, 52). Using a recombinant adenovirus expressing the green fluorescent protein (GFP) as a reporter, greater than 95% of myocytes express the transgene 48–72 h post-transfection. Cardiomyocytes were infected with vectors expressing FLAG-tagged wild-type p38α and p38β. Adenovirally encoded p38β has a higher apparent MW than p38α, thus enabling us to easily distinguish between the isoforms using p38 antibodies (**Fig. 7**). At a multiplicity of infection of 10, adenoviral-directed p38α and p38β expression was detected at comparable levels by Western blot analysis (Fig. 7*A*).

Using phospho-specific antibodies to examine activation, we noted that during ischemia p38α exhibits a strong phosphorylation similar to that seen with endogenous p38 in untransfected controls (see Fig. 7*B*). Preconditioning, which decreases endogenous p38 activation during ischemia, also significantly attenuates ectopically expressed p38α phosphorylation (P<0.05). In contrast, transfected p38β, which exhibits a high level of basal phosphorylation, is inhibited during ischemia (P<0.01) and this inhibition is moderately enhanced in preconditioned cells (Fig. 7*C*).

Effect of p38 isotype activation during simulated ischemia

As shown in Fig. 6 and elsewhere (22–24, 53), inhibition of p38 during ischemia using SB203580 protects against cell death. Since we demonstrate a selective activation of p38α over p38β during ischemia, we would expect that protection ensues as a result of p38α inhibition with SB203580. To test this hypothesis, we transfected cells with a dominant negative p38α mutant (TGY$^{180-182}$ to AGF: see Materials and Methods). As shown in **Fig. 8*A***, 48 h after transfection of either

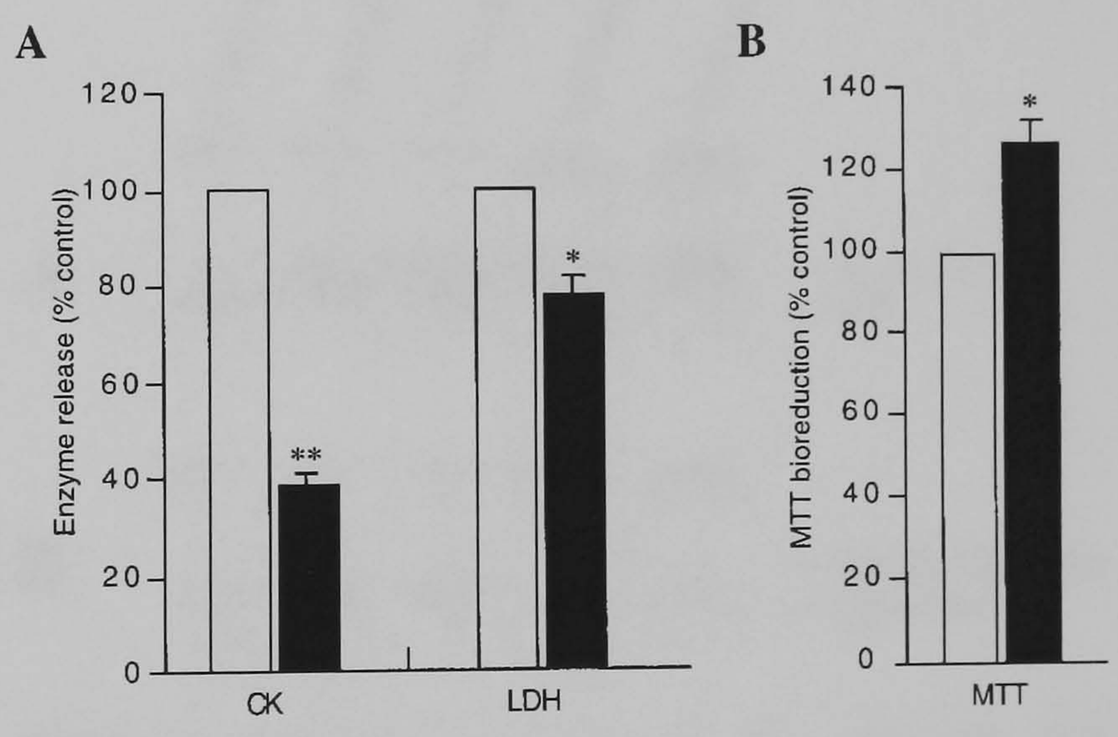

Figure 6. Cell viability after 6 h of simulated ischemia in the presence of SB203580. *A)* Total CK and LDH released into ischemia buffer during 6 h of simulated ischemia. Panel *A* therefore represents cell injury during ischemia alone. *B)* Cell viability was measured by MTT bioreduction within monolayers after 6 h simulated ischemia and 2 h reoxygenation. Injury in the presence of SB203580 (■) is expressed as a percentage of injury in the absence of SB203580 (□). Viability in cells treated with SB203580 alone for 6 h (without ischemia) was equivalent to that observed in untreated cells (results not shown). All P values are for comparisons between ischemia in the presence and absence of SB203580. *P<0.05, **P<0.001, n=20.

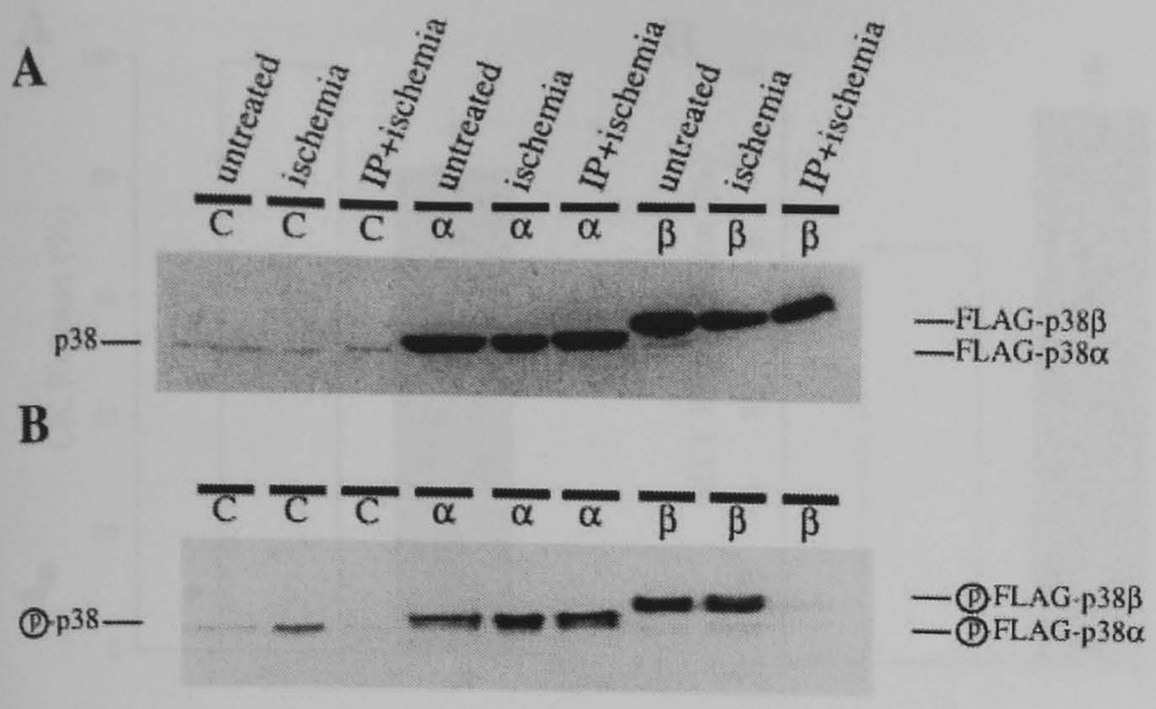

Figure 7. Activation of p38 isotypes during ischemia in untreated and preconditioned cells. Cardiocytes were infected with adenoviral constructs encoding FLAG-tagged wild-type p38-α or -β. 48–72 h postinfection untreated and preconditioned cardiocytes were subjected to 2.5 h ischemia before cells were harvested and lysed for Western blots. *A)* The relative expression of p38 signaling molecules in infected cells were compared with untreated cells using anti-p38 antibodies. *B)* Identical lysates were probed with dualphospho-specific p38 antibodies to detect isotype-specific p38 activation during ischemia. *C)* Mean p38 activation during ischemia compared to untreated controls in at least 4 independent experiments. $^{\S}P<0.001$, p38α activation vs. p38β activation during ischemia. $^{*}P<0.05$, p38α activation during ischemia with vs. without preconditioning (IP). $^{\P}P<0.01$, p38β activation during ischemia vs. nonischemic/untreated cells.

wild-type or dominant negative p38α (p38αDN), comparable overexpression of p38 can be detected. After 2.5 h ischemia, which is the time of maximal p38 activation, cells expressing p38αDN showed no significant p38 activation whereas cells expressing wild-type p38α showed increased p38 activation (Fig. 8*B*). Moreover, the cells expressing p38αDN were protected against simulated ischemia compared to empty vector transfected cells (CK release=82.9±3.9% and MTT bioreduction=130.2±6.5%, $n=8$, $P<0.05$, see **Fig. 9**). Expression of wild-type p38α had no effect on cell injury (Fig. 9).

In summary, our data suggest that simulated ischemia selectively activates ectopically expressed p38α over p38β ($P<0.001$). One consequence of this activation in our system is a decrease in myocyte survival. Constitutively active PKC-δ, which renders myocytes resistant to ischemia, inhibits ischemia-induced p38α activation. An identical effect occurs in untransfected cells preconditioned by brief simulated ischemia. Similar inhibition of p38α with a dominant negative mutant also protects against ischemia. These data suggest that the protection conferred by preconditioning may, at least in part, be mediated through a reduction in ischemia-driven p38α activation.

DISCUSSION

Experimental findings

We have investigated the relationship between preconditioning, protein kinase C and mitogen-activated protein kinase in primary cultures of neonatal cardiocytes. Our data show that during lethal simulated ischemia, p38α-MAPK exhibits a period of prolonged phosphorylation that reduces myocyte survival. In addition, preconditioning and the expression of active PKC-δ inhibit p38α activation during lethal ischemia and enhance myocyte survival. Inhibition of p38α activation with SB203580 or dominant negative p38α (p38αDN) also gives rise to protection. These findings suggest that p38α-MAPK modulates cardiocyte survival during simulated isch-

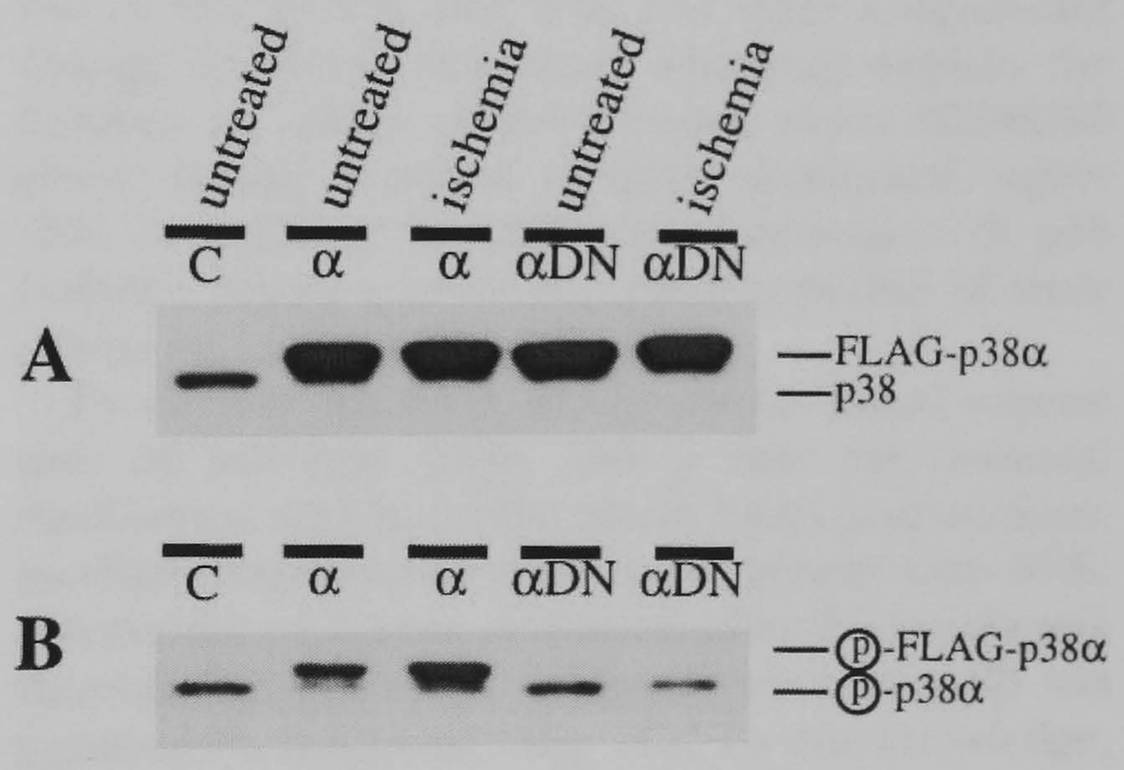

Figure 8. Modulation of ischemic p38 activation with p38αDN. Cardiocytes were infected with adenoviral constructs encoding FLAG-tagged dominant negative or wild-type p38α. 48 h postinfection, cells were subjected to 2.5 h simulated ischemia or left untreated. Myocytes were then harvested and lysed for Western blot analysis. *A)* The total p38 levels were assessed with anti-p38 antibodies. Adenoviral infection causes a consistent level of expression of either wild-type or dominant negative p38α that is unaltered during ischemia. *B)* Activation of p38 was assessed using phospho-specific p38 antibodies. Ischemia caused a marked activation of p38 in wild-type-p38α transfected cells, whereas p38 activation decreased during ischemia in cells expressing dominant negative p38α.

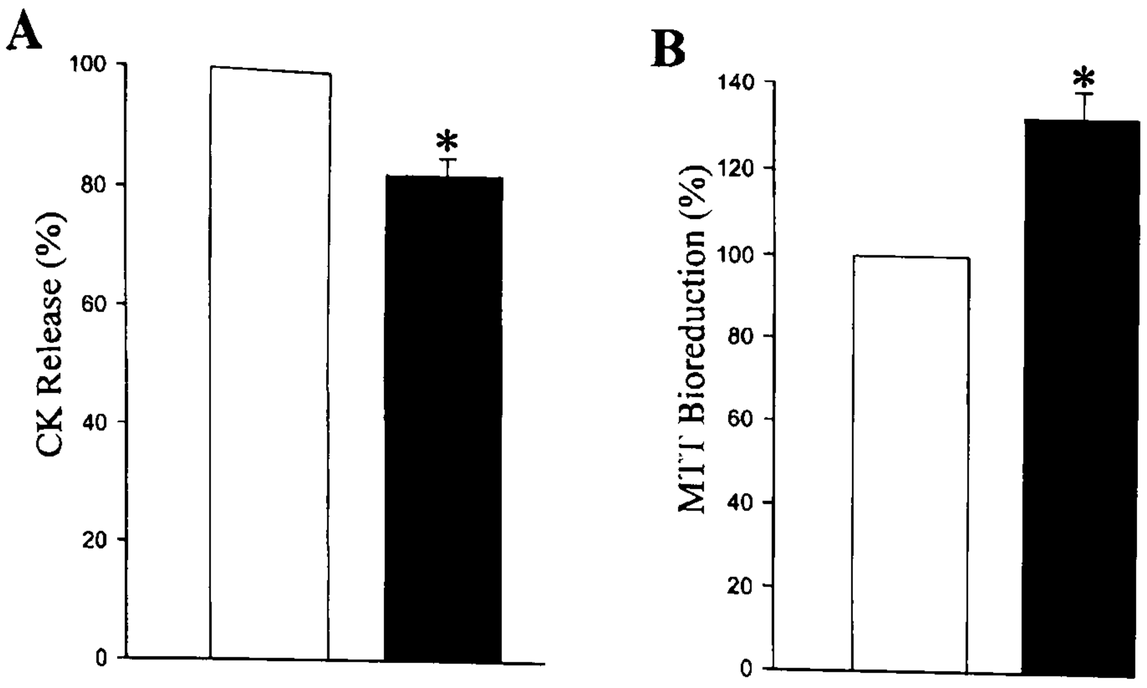

Figure 9. Effect of p38α activation on cell survival during ischemia. Myocytes transfected as in Fig. 8 were exposed to simulated ischemia for 6 h. Panel *A* therefore represents cell injury during ischemia alone. *B)* Cell viability was measured by MTT bioreduction within monolayers after 6 h simulated ischemia and 2 h reoxygenation. Injury in the presence of p38αDN (■) is expressed as a percentage of injury in the presence of an empty vector (□). Viability in cells treated with p38αDN alone for 6 h (without ischemia) was equivalent to that observed in untreated cells (results not shown). All *P* values are for comparisons between ischemia in the presence of p38aDN vs. empty vector. *$P<0.05$, $n=8$.

emia, which may represent a new and specific therapeutic target.

Signaling during preconditioning and ischemia

Previous reports suggest that activation of p38 during preconditioning is responsible for the resulting protection (20, 54, 55). These conclusions were based on the ability of SB203580 to inhibit protection when given during preconditioning. If p38 activation during preconditioning occurs downstream of PKC, then we would expect overexpression of active PKCδ, which we have shown protects myocytes during ischemia (29), to have activated the p38 pathway. Our findings do not support this hypothesis, since p38 activation was inhibited rather than activated by active PKC-δ. Expression of active PKC-ε, another PKC isoform implicated in preconditioning (56), also inhibits p38 activation during simulated ischemia (results not shown). These findings support a protective role for PKC activation during lethal simulated ischemia since all studies addressing the role of p38 during ischemia, in the absence of preconditioning, demonstrate that activation is detrimental, with SB203580 decreasing infarct size and enhancing postischemic functional recovery (23, 24).

The effect of preconditioning on p38 signaling during ischemia

Overexpression of active PKC mutants does not necessarily mimic physiological preconditioning, so we wanted to examine the effect of ischemic preconditioning on p38 activation during ischemia. As shown in Fig. 5, preconditioning significantly inhibited p38 activation during ischemia. Of course, the basis of this inhibition could be a consequence rather than a cause of protection. If a cause of protection, one would expect p38 inhibition to protect during ischemia. As shown in Fig. 6 and in agreement with other reports (22–24), inhibition of p38 with SB203580 does protect against ischemic cell death. This is the first data, to our knowledge to document a mechanistic link between ischemic preconditioning and reduced p38 activation during ischemia.

The contribution of p38 isoforms

The limitations of this work became apparent when considering recent reports of various p38 isotypes, two of which (p38α and β) are highly expressed in the heart. The likelihood that these isotypes carry out different, perhaps even opposite, intracellular functions casts doubt over the use of nonselective inhibitors (SB203580) and antibodies to infer the function of p38. Neglecting isoform-specific effects could lead to contradictory results. For example, a treatment such as preconditioning may switch the balance of activation from one isoform to another, which, if they have opposing roles may have a dramatic effect on cell fate. But the detection of p38 phosphorylation using antibodies would be insensitive to this switch, and thus not show a significant change in overall activation. This may explain the findings in rabbit cardiomyocytes when SB203580 given during ischemia actually accelerates injury (57). Examining the differential activation of p38 isoforms allows a more complete appraisal of their effects on cell viability.

To do this, we used adenoviral-mediated expression of wild-type p38α and β into rat neonatal cardiocytes, which, unlike whole heart preparations, produced transfection efficiencies greater than 95%. Assessment of isotype activation after 2.5 h ischemia showed that p38α was activated, whereas p38β was significantly inhibited (Fig. 7*C*). To our knowledge, this is the first demonstration of differential activation of p38 isoforms by a physiological stress. According to Wang and co-workers, p38α activation in cardiac myocytes is sufficient to cause apoptosis and cell death, whereas p38β is responsible for hypertrophy and survival (52). In our model, this hypothesis would fit with a mechanism whereby p38α is the specific detrimental MAPK isoform activated by ischemia. Preconditioning, which inhibits p38, also attenuates the p38α pathway during ischemia, which should account at least in part for the associated protection (Fig. 7*C*).

The consequence of p38α activation during ischemia

If decreased p38α activation does contribute to protection, then inhibition of this pathway should be sufficient to protect. Although SB203580 has been shown to protect, both in this study (Fig. 6) and others (22–24), it inhibits both p38α and p38β. Moreover, recent reports have questioned its specificity since it can inhibit (58, 59) and even activate (60) other kinases.

We used dominant negative p38 mutants to specifically inhibit the activation of individual isoforms during ischemia. Dominant negative p38α decreased endogenous p38 activation during ischemia, which may reflect a decrease in endogenous p38β activation (Fig. 8*B*). This inhibition of p38α caused an increase in cell viability (Fig. 9), whereas the presence of p38βDN had no effect (results not shown).

In summary, sustained p38 activation occurs during lethal simulated ischemia in cultured rat neonatal cardiocytes. This activation can be attenuated by cardioprotective treatments such as preconditioning and overexpression of active PKC-δ. Moreover, our results support the concept that p38α and p38β are differentially regulated during ischemia, since ischemia is accompanied by an increase in p38α and a decrease in p38β phosphorylation. In addition, specific inhibition of p38α activation, but not p38β, is protective. Taken together, these observations suggest that the inhibition of p38α activation during prolonged ischemia is the cause rather than the consequence of preconditioning. Ultimately, understanding the signaling mechanisms that modulate ischemic cell injury will facilitate novel interventions that preserve ventricular function by reducing the rate of necrosis during myocardial infarction. FJ

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